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**GLYCATION AND THE MAILLARD
REACTION IN VITRO - IMPLICATIONS FOR
DIABETES MELLITUS**

**Thesis submitted for the Degree of Doctor of Philosophy
in the Discipline of Biology
OPEN UNIVERSITY**

by

Nessar Ahmed B.Sc. (Hons)

Oxford Research Unit

Date of submission : 4th May 1992

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This Thesis is Dedicated to

MY PARENTS

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I am also grateful for the technical support provided during the course of this work by various members of the Oxford Research Unit notably Alan Knight, Ted Beaver and Jason Shackleton. I am particularly indebted to Professor Gerald Elliott for helping me to secure funds for my overseas visits. I would like to give a special mention to my family who have been a constant source of inspiration and encouragement throughout the duration of this thesis.

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Abstract

A colorimetric microassay for protein glycation based on the periodate method has been developed. This microassay has improved sensitivity, speed and yield of chromophore enabling it to be used on 0.1mg aliquots of intact protein. However this assay is unsuitable for collagen and so a second microassay based on the TBA method was developed. This TBA microassay has an improved yield of HMF, is less cumbersome, requires less protein than earlier versions, and is suitable as a rapid assay for glycated collagen.

Fructation induces AGE formation considerably faster than glucation in vitro probably because of the highly reactive fructose-derived aldehydic AP. However glycation assays designed for the glucose-AP (a ketose) were found to underestimate the true extent of fructation. For this reason a microassay based on the DNPH method was developed to quantify fructation.

Comparative rates of protein fluorescence generated by different sugars were identical to their comparative rates of SB formation but not crosslinking. Using model proteins and different sugars, this study has revealed that fluorescent-AGE and crosslinked-AGE may be formed by independent pathways ie some proteins are more susceptible to crosslinked-AGE and vice versa. Phosphate, pH and calcium have been found to increase glucose and fructose-derived fluorescent-AGE in BSA.

Contrary to current dogma, no evidence for an accelerated rise in AGE levels in reincubated proteins after removal of free sugars was found. Only a small increase in fluorescence of reincubated glucated-BSA but not fructated-BSA was detectable. These studies suggest that free sugars have a significant role in fluorescence generation and indeed using chemically modified proteins, evidence has been presented for a reaction between the

AP and free sugars to form fluorescent products. This secondary glycation is more pronounced for fructose and explains in part the higher fluorescence generated by fructated proteins.

Comparative studies between inhibitors with different modes of action have revealed that aminoguanidine is the most effective at reducing fluorescence and crosslinking of proteins in vitro on a molar basis. This is probably because the compound has multiple sites of action and more than one amino group. Phenylenediamine reacts with 3-DG preventing formation of AGE and was found to have a greater inhibitory effect on fructose compared to glucose-derived AGE.

In this study, no evidence was found for sugar-induced protein fragmentation in vitro unless transition metals were included. Furthermore binding of the transition metal to protein appears to be important for significant fragmentation to occur and in vivo this autoxidative glycation may be restricted to the limited number of copper binding proteins only.

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Abbreviations

The following abbreviations were used throughout the text;

A ₂₇₉	Absorbance at 279nm
AGE	Advanced glycation endproduct
AP	Amadori product
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
°C	Degrees celcius
cm	Centimetre
CML	Carboxymethyllysine
Cont	Control
CV	Coefficient of variation
DETAPAC	Diethylenetriaminepentaacetic acid
DDL	Diacetyldihydrolutidine
3-DG	3-Deoxyglucosone
DNPH	Dinitrophenylhydrazine
EA	Erythronic acid
EDTA	Ethylenediaminetetraacetic acid
em	Emission
ELISA	Enzyme linked immunosorbent assay
ex	Excitation
F-AP 1	Fructose-Amadori product 1
F-AP 2	Fructose-Amadori product 2
FDR	Formaldehyde detection reagent
FFI	Furoyl-Furanyl-Imidazole
FL	Fructoselysine
Fruc	Fructose
g	Gravitational field
GA	Glyceric acid

Gal	Galactose
Gluc	Glucose
Hb	Haemoglobin
HEPES	Hydroxyethylpiperazine-ethanesulphonic acid
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HSA	Human serum albumin
KD	Kilodalton
LDL	Low density lipoprotein
LJM	Limited joint mobility
LL	Lysino-lactic acid
M	Molar
mm	Millimetre
MFP-1	Maillard fluorescent product 1
mg	Milligram
mins	Minutes
ml	Millilitre
mM	Millimolar
Mw	Molecular weight
mwco	Molecular weight cut off
NALMA	N-Acetyl-L-lysine-N-methyl amide
NBT	Nitro blue tetrazolium
nm	Nannometre
NMR	Nuclear magnetic resonance
nmols	Nannomoles
Pa	Pascals
PAGE	Polyacrylamide gel electrophoresis
PBA	Phenylboronate agarose
PD	Phenylenediamine

r	Correlation coefficient
Rib	Ribose
RM	Reductively methylated
RNA	Ribonucleic acid
RNase	Ribonuclease
s	Seconds
SB	Schiff base
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFA	Serum fructosamine
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N, N, N',N'-Tetramethylethylene-diamine
TNBS	Trinitrobenzene sulphonic acid
ul	Microlitre
ug	Microgram
uM	Micromolar
UV	Ultra violet
V	Volt
v/v	Proportion by volume
w/v	Proportion by weight

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CHAPTER 1

Introduction

Incubating proteins with reducing sugars for prolonged periods of time results in the solution becoming yellow brown in colour. This reaction is referred to as the Maillard or browning reaction and involves an initial reaction between sugar and protein called glycation. Once a protein is glycated, it undergoes subsequent reactions to form poorly characterised structures called advanced glycation endproducts or Maillard products.

1.1: Historical Review of the Maillard Reaction

In 1908, an Englishman called Ling first proposed that sugars may react with proteins to produce a characteristic yellow brown colour (1). However, it was Maillard in France who provided the first experimental evidence for this reaction in 1912 (2). He did this by heating various amino acids with glucose and found that the liquid turned yellow, then rapidly deep brown and eventually dark brown polymers called melanoidins were formed. As early as 1912, Maillard predicted that this reaction may be of significance in vivo. However, his findings were ignored by medical scientists for over 50 years, and instead became of great interest in food science where the reaction is not only important for the production of taste, aroma and colour in foods but can also cause loss of their nutritive value. In 1931, Amadori described two products ie a stable and unstable isomer formed during the initial stages of the Maillard reaction (3). Weygrand in 1940 demonstrated that the labile isomer or aldimine is formed first and rearranges to form the stable ketoamine named the Amadori product (4).

Interest in the Maillard reaction in vivo began when Rahbar in 1968 showed that diabetic patients had elevated amounts of a fast moving haemoglobin (Hb) on electrophoresis (5). This Hb designated HbA_{1c} comprises about 4%

of the total Hb in the red blood cell. In the same year, Bookchin and Gallop demonstrated that this fraction was "glycosylated" with a 1-amino-2-deoxyfructose molecule (or Amadori product) attached to the N-terminal valine of the chain (6). Fluckiger and Winterhalter demonstrated that the formation of HbA_{1c} occurs non-enzymatically because HbA_{1c} can be synthesised in vitro in the absence of any enzymes (7).

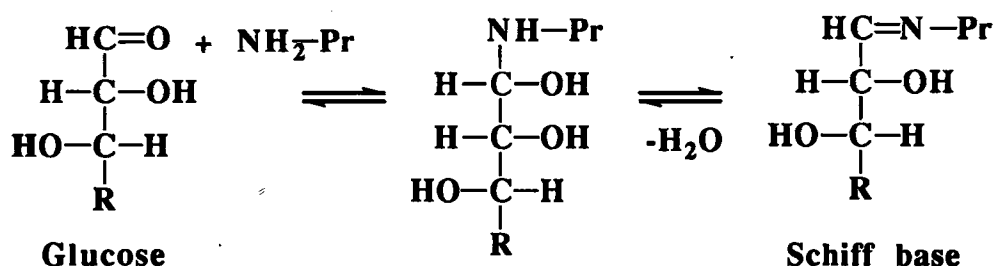
Interest in the role of the Maillard reaction in the secondary complications of diabetes and ageing was prompted by the discovery of glycated collagen (8), and the observation that complications occur preferentially in tissues which do not require insulin for glucose transport.

1.2: Non-Enzymatic Glycosylation (Glycation)

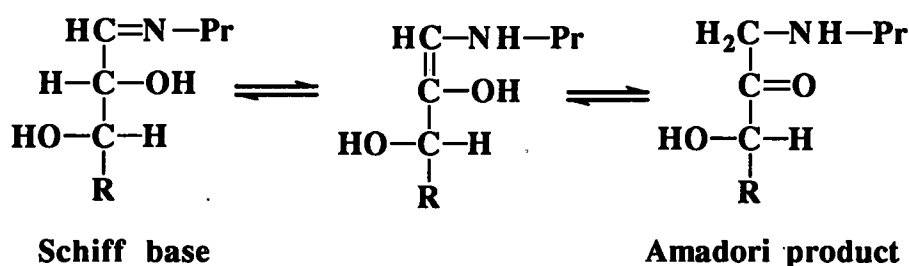
Glycation is also referred to as non-enzymatic glycosylation to distinguish it from enzymatic glycosylation which occurs in the endoplasmic reticulum during the synthesis of glycoproteins. Such glycoproteins consist of an oligosaccharide chain linked via N-acetylglucosamine to an asparagine residue (N-glycoside), or an N-acetylgalactosamine to a serine or threonine residue from protein (O-glycoside). This enzymatic glycosylation is highly regulated and controlled by enzymes called glycosyl transferases. In contrast, glycation is a spontaneous reaction between reducing sugars in the acyclic form and free amino groups from proteins. Glycation refers to a reaction between any reducing sugar and protein whereas terms such as glucation, fructation and ribation are used to specify reaction with glucose, fructose and ribose respectively.

The oxygen in the carbonyl group has a higher electronegativity than the carbon and so the electrons are pulled towards it making the C atom electron deficient and the bond is said to be polar. Nucleophilic attack occurs on the C atom by reagents which are electron rich such as amino groups since they have a lone pair of electrons.

The glycation reaction starts with a nucleophilic addition reaction between the carbonyl group of reducing sugar and free amino group of protein to form a labile aldimine or Schiff base (SB).



This first step is freely reversible and the SB exists as an equilibrium between the acyclic and pyranosyl or ring form (9). Inevitably, some of the SB rearranges to a more stable ketoamine ie the Amadori product (AP).



This second step is also reversible but only to a limited extent. For most of the time the AP exists in its ring structure ie the β -furanose and β -pyranose forms. A ^{13}C -NMR study using glucated RNase and polylysine has demonstrated that at equilibrium 14% of the glucose is attached as the pyranose form of SB and the remainder as the ring form of AP, mostly the β -pyranose form (9). The glycation reaction between glucose and a protein (glucation) is outlined in fig 1.1. The relative orientation of substituent groups will clearly be different in furanosyl and pyranosyl conformers. This could affect noncovalent interactions between glycated proteins and other macromolecules, but will have no bearing on covalent reactions of the glycation sequence, since these all involve the sugar derived carbonyl. This

carbonyl is only available in the acyclic form. The conversion of protein to SB is freely reversible, although in the continued presence of free sugar the equilibrium is displaced heavily in favour of SB. Under these circumstances, the equilibrium constant is 3.9 M^{-1} for glucated albumin and 2.7 M^{-1} for glucated Hb; the respective dissociation rate constants are 0.39 and $0.33 \text{ M}^{-1}\text{h}^{-1}$. Conversion of SB to AP is essentially irreversible (186).

1.3: Rates of Reaction Between Sugars and Protein

A number of factors have been shown to affect the rate of reaction between a protein and a particular sugar in vitro ;

- 1: concentration of sugar
- 2: duration of exposure to sugar
- 3: temperature
- 4: pH
- 5: phosphate concentration

In vivo, the extent of glycation will depend on the degree and duration of hyperglycaemia, since the other factors remain constant.

The initial rate of reaction between different sugars and a protein depends on three major factors;

- 1: the type of carbonyl group ie whether aldose or ketose
- 2: the percentage of reducing sugar in the acyclic form
- 3: the number of carbon atoms in sugar

The carbonyl groups of aldoses are more polar than those of ketoses and so aldoses tend to react approximately 50 times faster than ketoses (10, 11). Sugars also differ in the proportion of molecules in the acyclic form and this is important since only molecules in the acyclic form can react with free

amino groups. Table 1.1 lists the reaction rate of different sugars with Hb. A higher proportion of fructose (0.7%) exists in the acyclic form compared to glucose (0.002%) and so would be expected to react some 350 times faster than glucose. However, glucose is an aldose and approximately 50 times more reactive than fructose. Taking both factors into account, fructose would be expected to react approximately 7 times faster than glucose. This agrees well with the experimental value obtained in table 1.1. As the sugar molecule takes part in glycation, more of the ring form may convert to the acyclic form and this rate of interconversion may be important but has not been investigated.

The number of C atoms in the sugar are also important and in vitro studies have shown that trioses react faster than pentoses which in turn react faster than hexoses (11). This is probably because smaller sugar molecules have greater accessibility to free amino groups and a higher proportion exist in the acyclic form.

Certain groups within the protein structure are more susceptible to glycation than others, for eg Bunn et al have demonstrated that the more reactive lysines in Hb are located adjacent to acidic amino acids (12). Since the Amadori rearrangement is an acid catalysed reaction, proton donors proximal to these groups could greatly increase glycation. Furthermore Iberg and Fluckiger noted that reactive lysine residues in albumin are in Lys-Lys, Lys-His, Lys-Lys-Lys and Lys-His-Lys sequences (13).

1.4: Fate of Glycated Proteins

A glycated protein can enter several different pathways as shown in fig 1.2;

The glycated protein may;

- 1: be turned over ie catabolised
- 2: reconvert to the SB which can dissociate to give free protein
- 3: be oxidized to give inert compounds
- 4: undergo further reactions, which may include oxidation to form AGE

1.5 Oxidation of Glycated Proteins

The AP can be oxidized to form;

- 1: Carboxymethyllysine (CML) and Erythronic acid (EA) or
- 2: Lysino-lactic acid (LL) and Glyceric acid (GA)

The CML and EA are formed by oxidative cleavage between C2 and C3 of AP (14) whereas the LL and GA are formed by a split between C3 and C4 (15).

In vitro work using an analogue of glycated lysine called N-formyl-N-fructoselysine has demonstrated that CML and LL formation requires oxygen and the major product formed is CML. Free radical scavengers inhibited production of CML and LL suggesting that a free radical mechanism is involved. Fig 1.3 shows formation of CML and LL from fructoselysine (FL).

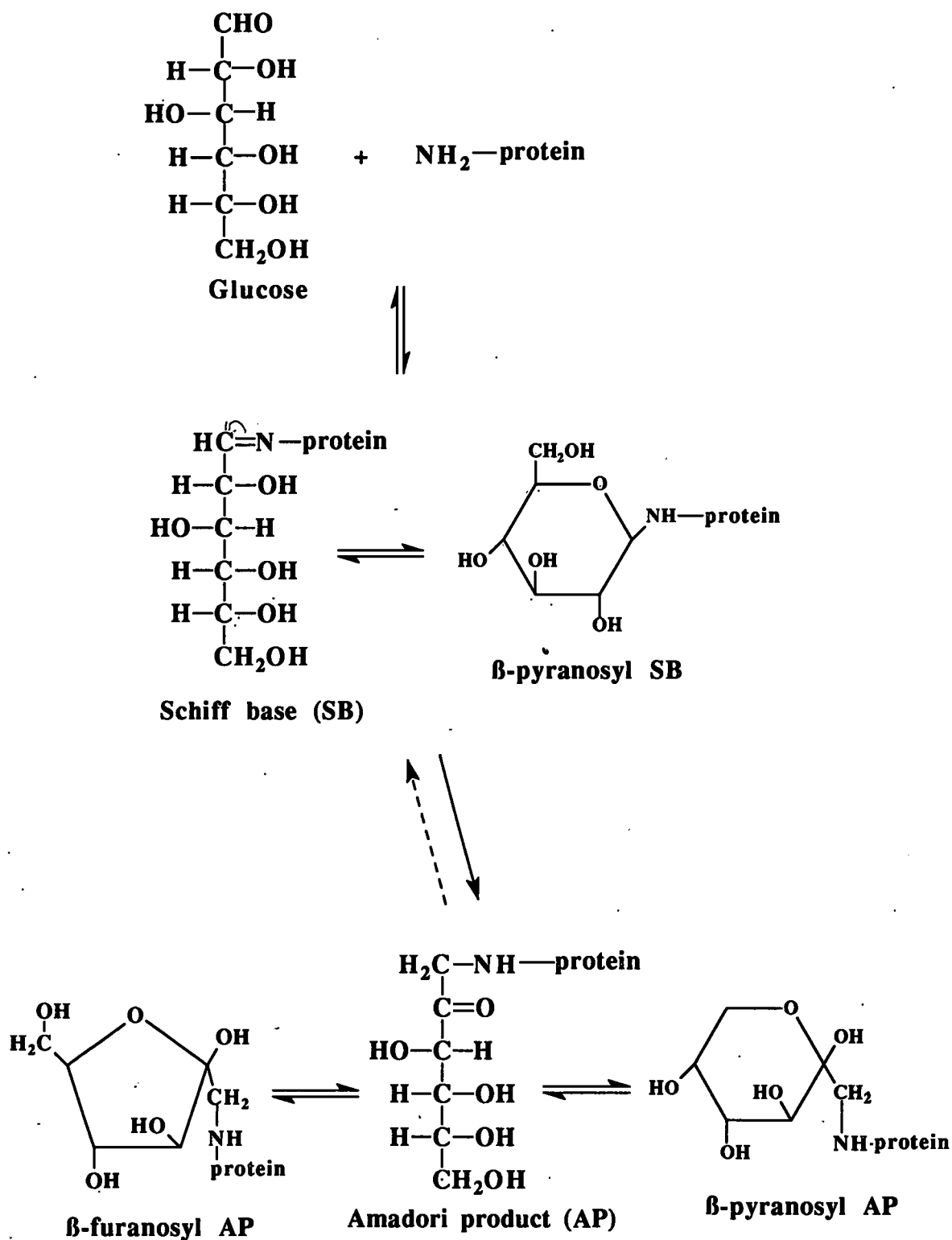


Fig 1.1: Glycation of a protein by glucose (glucation)

Sugar	Type	Carbonyl	% Acyclic	Rel reaction
Glucose	Hexose	Aldose	0.002	1
Fructose	Hexose	Ketose	0.700	7.5
Galactose	Hexose	Aldose	0.020	4.6
Xylose	Pentose	Aldose	0.020	4.8
Ribose	Pentose	Aldose	0.040	16.6
Xylulose	Pentose	Ketose	8.000	25

Table 1.1: Relative rates of reaction of various sugars with haemoglobin (adapted from ref (169)).

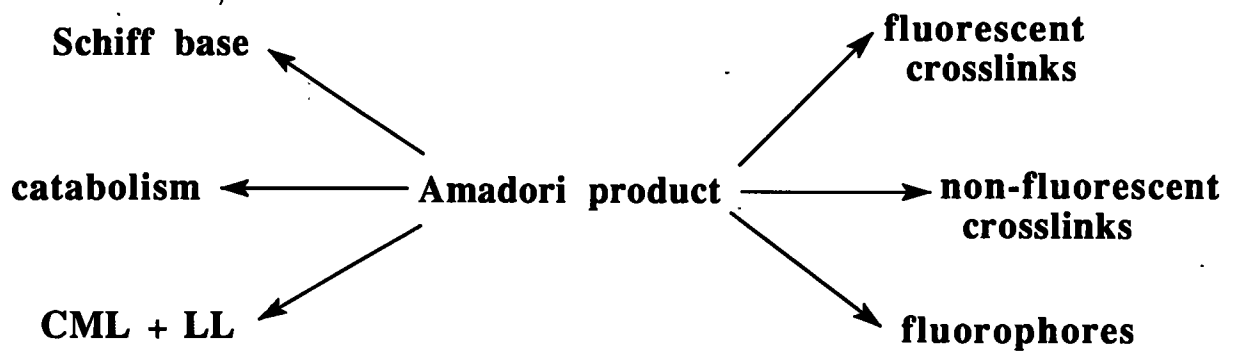


Fig 1.2: The various pathways that a glycated protein may enter

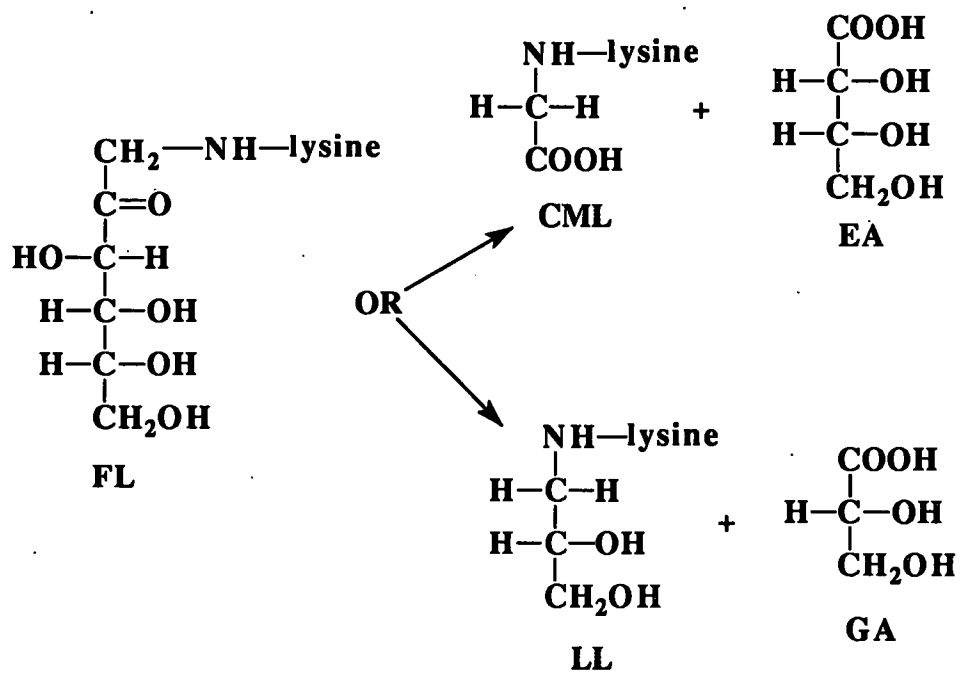


Fig 1.3: Oxidative degradation of FL to give CML and LL

1.6: Advanced Glycation Endproducts

Once a protein is glycated, it can go on to form advanced glycation endproducts (AGE) by reactions between the AP and other AP's or free amino groups from the same or different protein. AGE form slowly and since proteins are continually turned over, only long lived proteins will accumulate them to any great extent in vivo. The limited information available on the structure of AGE suggests that they may be;

- 1: fluorescent crosslinks
- 2: non-fluorescent crosslinks
- 3: fluorophores

The latter are fluorescent groups attached to proteins which may be capable of forming crosslinks. Formation of AGE is believed to involve reactive dicarbonyl intermediates called glucosones. Kato in 1961 first isolated glucosones as intermediates in the Maillard reaction in model systems. Glucosones are formed by degradation of the AP regenerating the free amino group and there are three different types (reviewed in (16)). Of the three glucosones, 3-deoxyglucosone (3-DG) shown in fig 1.4 has received the most attention and is the most important with respect to pyrrole formation (17) and protein crosslinking (18). A possible pathway for formation of 3-DG may be similar to that proposed by Anet in 1960 (159). The AP or fructoselysine may react with free sugar to form a difructoselysine compound which may dissociate to give fructoselysine and 3-DG (see fig 1.4). However the precise mechanism for 3-DG formation is not yet known and it is now believed that a free amino group is generated after 3-DG production from an AP which is not the case in the pathway shown in fig 1.4 (16).

Chemical characterisation of AGE has proved difficult because of their complexity, low yields, their destruction during acid hydrolysis and generation of artefacts during their isolation. However, despite these difficulties three different types of AGE have been reported in the literature.

1.6.1: Furoyl-Furanyl-Imidazole (FFI)

There is only controversial evidence for the existence of this AGE believed to be formed from two AP's and characterised by Pongor et al (19) in 1984. FFI was extracted into chloroform as a fluorescent pigment from glucated bovine serum albumin (BSA) and poly-L-lysine after they had been acid hydrolysed and alkalisied in ammonia. This pigment was purified by silica gel chromatography and its structure determined by NMR, mass spectrometry and chemical analysis. However, in 1988 Njoroge et al demonstrated that FFI is an artefact formed during the extraction process (20). Furosine is produced during acid hydrolysis of glycated proteins and in alkaline solutions is converted to furoyl glyoxal which can react with ammonia to form FFI. Using ^{15}N labelled ammonia, this group showed that the nitrogen atoms in FFI were derived from ammonia and not amino groups. The structure of FFI is given in fig 1.5.

1.6.2: Pyrraline

Four pyrrole compounds have been isolated from model systems by reacting neopentylamine with glucose under physiological conditions (21). All of these pyrroles have been detected previously in reaction mixtures at higher temperatures (22, 23) and all possess a carbonyl group which could be capable of reacting further to form protein crosslinks. A polyclonal antibody has been raised to one of these pyrroles named pyrraline (fig 1.6) and used to develop an ELISA (24). This immunological approach was necessary since acid hydrolysis destroys the compound. Using the ELISA, elevated pyrraline levels have been detected in albumin from diabetic

subjects ie 40 pmol/mg compared to 28 pmol/mg from controls. Although the mechanism for pyrraline formation is not yet known, it is believed to be formed by a reaction between free amino groups and 3-DG.

1.6.3: Pentosidine

In 1989, a fluorescent crosslink was isolated from the dura mater of ageing individuals and subsequently characterised by proton NMR and fast atom bombardment (25). This AGE named pentosidine consists of an arginine residue linked to a lysine residue via a pentose group as shown in fig 1.7. Pentosidine has been synthesised in vitro by incubating pentoses with equimolar amounts of lysine and arginine or with collagen. More recent studies have shown that pentosidine can be formed by reacting proteins with glucose, fructose and ascorbate in the presence of oxygen (26, 27). Furthermore, pentosidine has been shown to account for less than 1% of non-disulphide crosslinks in glucated lysozyme or RNase suggesting that it is not a major sugar-derived crosslink (26). Accumulation of pentosidine occurs in tissue collagens with age and at an accelerated rate in diabetes (28). Trace amounts of pentosidine have been found in human lens protein where its concentration increases with age (26). Another fluorescent crosslink named Maillard Fluorescent Product-1 (MFP-1) can form when proteins are incubated with glucose and accumulates in tissue proteins with age and diabetes in vivo (29). MFP-1 and pentosidine have similar absorbance and fluorescence spectra and recent findings suggest that they are the same compound (26).

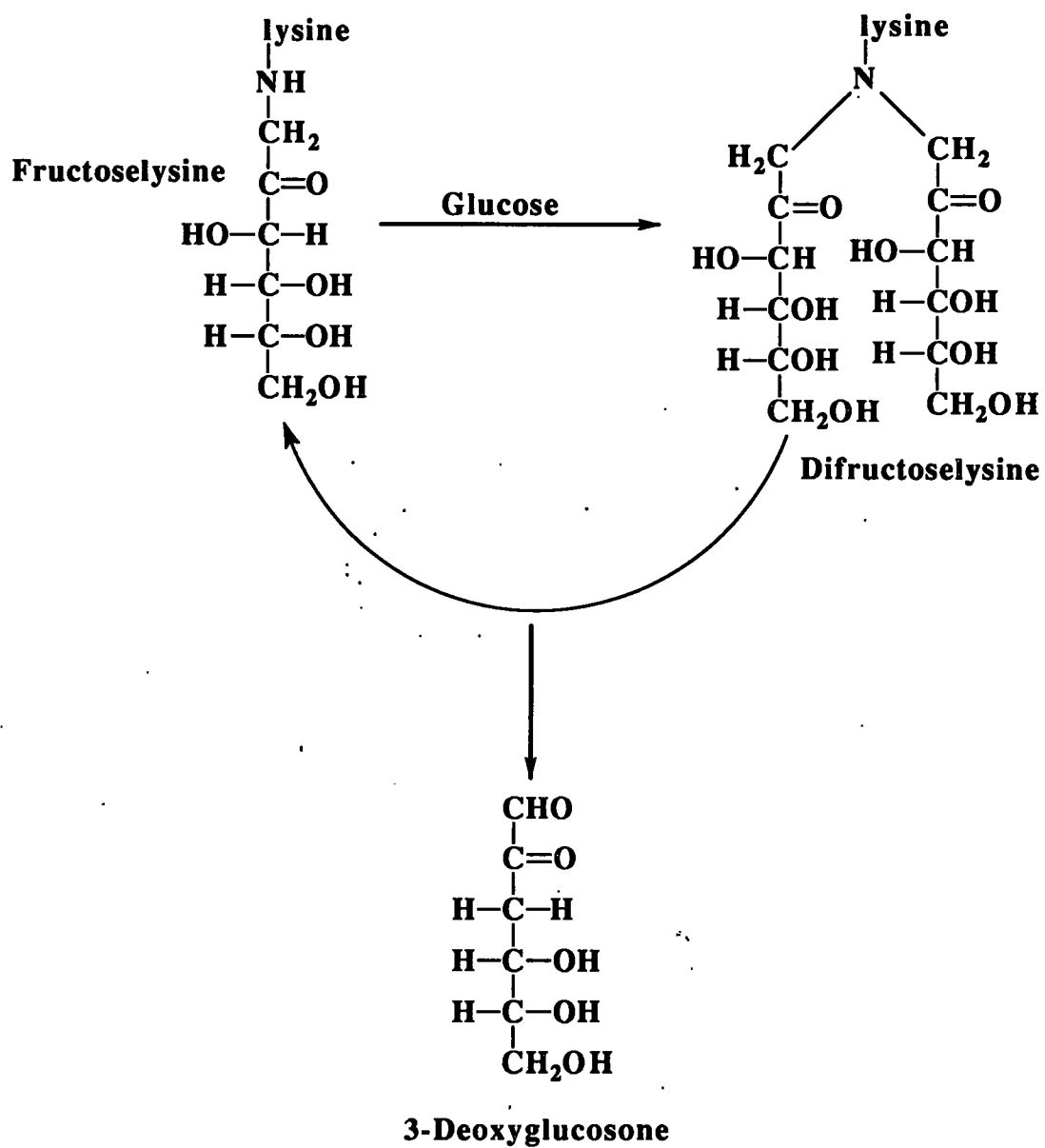


Fig 1.4: Possible pathway for 3-DG formation from an Amadori product

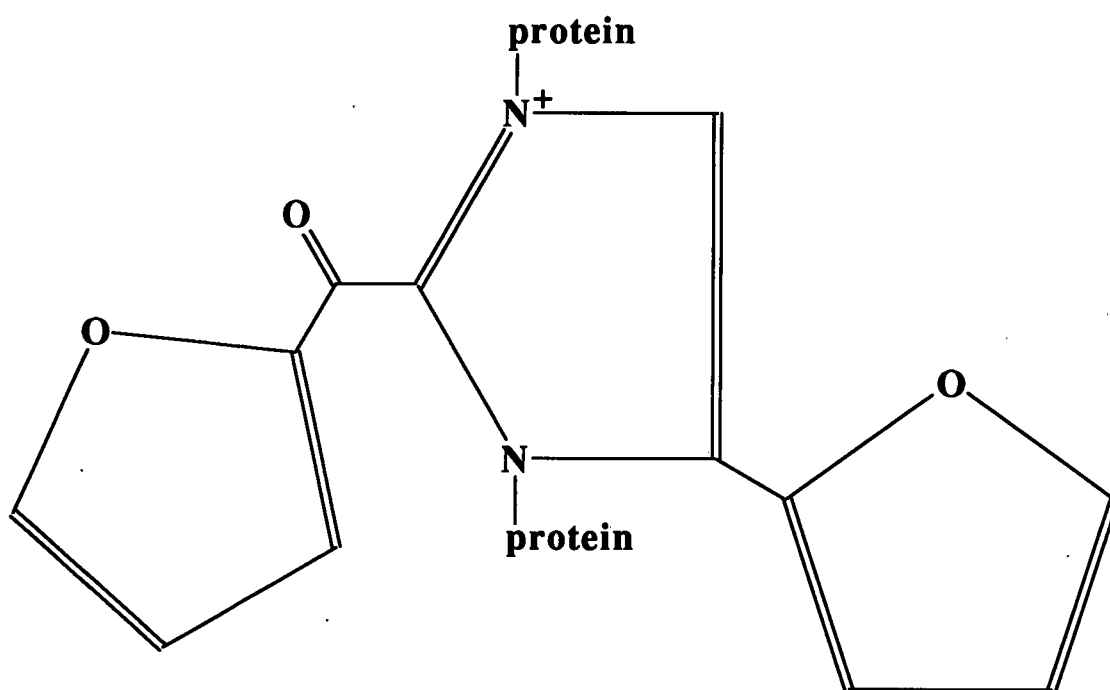


Fig 1.5: Chemical structure of Furoyl-Furanyl-Imidazole

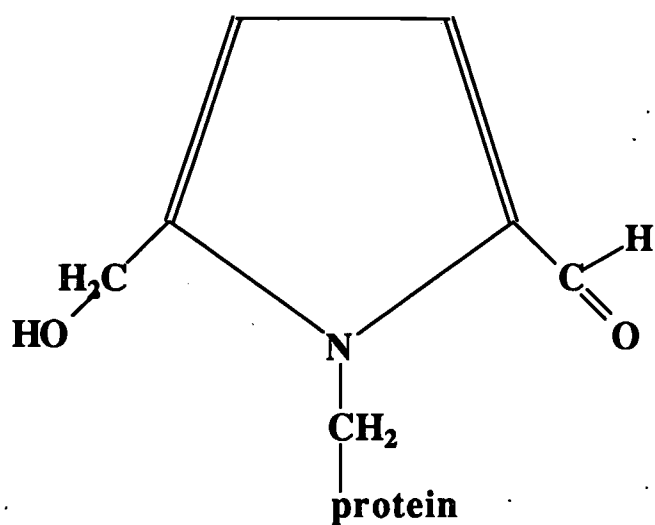


Fig 1.6: Chemical structure of pyrroline

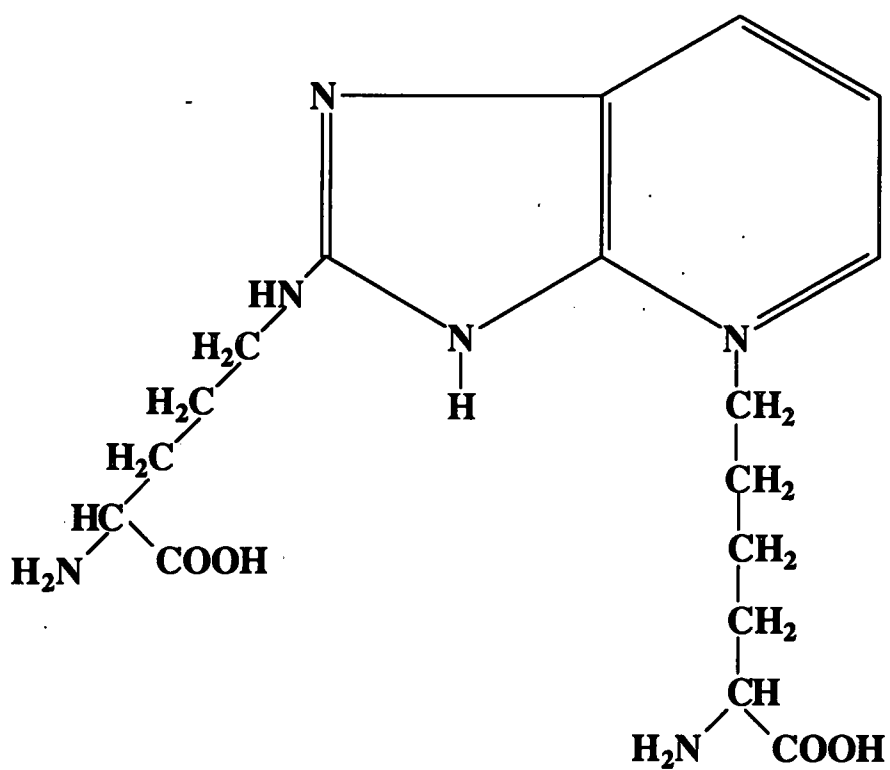
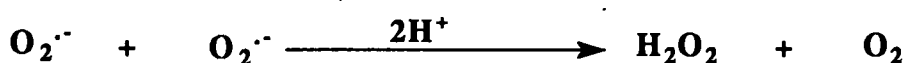


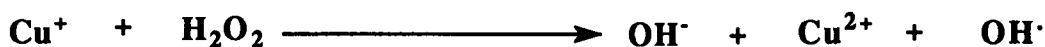
Fig 1.7: Chemical structure of pentosidine

1.7: Autoxidative Glycation and Free Radicals

Monosaccharides are known to exist in equilibrium with their enediol which can undergo autoxidation in the presence of transition metals to form an enediol radical anion (30). This enediol radical anion has an unpaired electron and reduces molecular oxygen to generate the superoxide anion radical ($O_2^{\cdot-}$). Two superoxide radicals can react to form hydrogen peroxide (H_2O_2) ie;



Nanomolar quantities of hydrogen peroxide have been detected during experimental glycation (31). A Fenton reaction may occur between this H_2O_2 and transition metals such as Fe^{2+} or Cu^+ to generate the highly reactive hydroxyl radical $OH\cdot$ ie;



Hydroxyl radicals generated from other sources for eg by gamma radiolysis of water are known to induce protein fragmentation (32), and this has led to the suggestion that fragmentation of proteins incubated in glucose is due to hydroxyl radicals generated by autoxidation of the sugar (33). Furthermore, hydroxyl radicals can also induce protein crosslinking and fluorescence particularly in the absence of oxygen. Some crosslinking does occur in the presence of oxygen but fragmentation is then much more pronounced (34). The metal chelators DETAPAC and EDTA inhibit protein fragmentation probably by sequestering the trace amounts of transition metal necessary for hydroxyl radical production (33). The superoxide radical is not as reactive as the hydroxyl radical and does not cause fragmentation (35). When forming the superoxide radical, the enediol radical anion itself is oxidized to form a dicarbonyl compound called a ketoaldehyde (36). The

process of autoxidative glycation is outlined in fig 1.8. Wolff et al have proposed that ketoaldehydes react with free amino groups to form structures called ketimines which are distinct from the ketoamines or AP and may participate in crosslinking and fluorescence (AGE) of proteins (36). This has led to the suggestion that antioxidants may be of therapeutic potential by inhibiting autoxidative glycation in vivo (33). However, this hypothesis has received considerable criticism since at present there is no evidence for ketimines in vivo or in vitro (37). Borohydride reduction of ketimines would form deoxyaminohexitols identical to those produced by ketoamines and therefore it would not be possible to distinguish between the products formed by glycation and those formed by autoxidative glycation. Whether ketoaldehydes formed by autoxidative glycation contribute significantly towards total monosaccharide attached to protein in vivo remains to be proved. It is noteworthy that levels of transition metals such as copper increase during diabetes (38) whereas plasma levels of antioxidants such as uric acid, vitamin C, vitamin E and glutathione decrease in diabetes (reviewed in (39)).

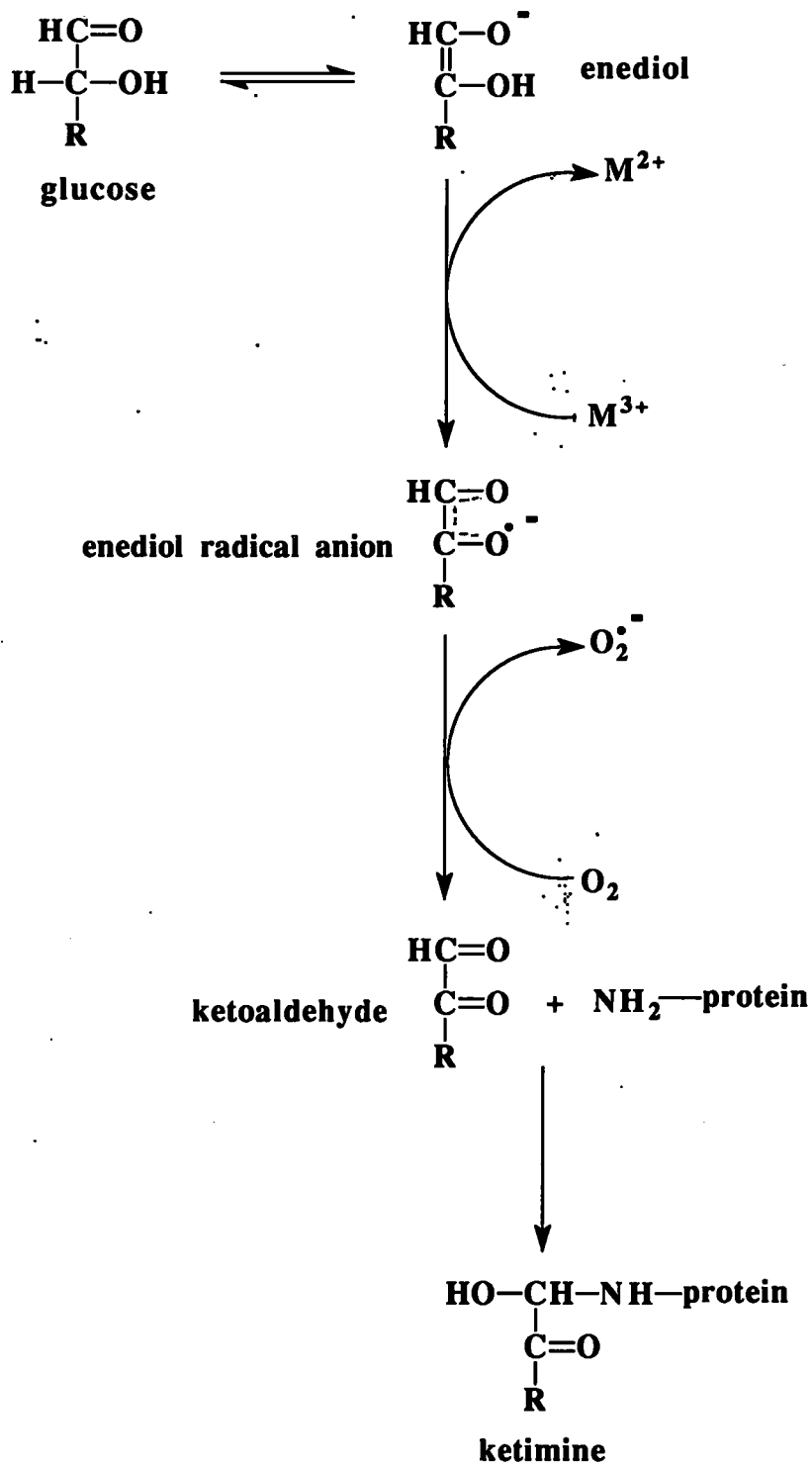


Fig 1.8: Autoxidative glycation of a protein

1.8: Measurement of Glycation

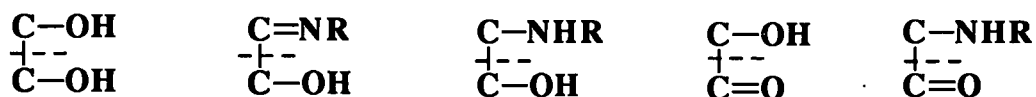
Assays for glycated proteins can be performed on intact proteins or protein hydrolysates, and those which detect the AP and not SB are preferred since the labile SB can dissociate to different extents giving rise to inconsistent results. Free sugars interfere in some assays and have to be removed by dialysis.

1.8.1: Thiobarbituric Acid (TBA) Assay

Mild acid hydrolysis of the AP form of glycated proteins causes release of hydroxymethylfurfural (HMF) (40). After removal of protein, loss of H₂O occurs and the HMF produced (fig 1.9) can be reacted with thiobarbituric acid (TBA) to form a chromophore which absorbs at 443nm. Alternatively, the HMF can be detected by its A₂₈₀ after separation by HPLC (41). The net production of HMF depends on its release and destruction during hydrolysis and so the assay is non-stoichiometric. However, it has advantages in that all AP's whether they are attached to alpha or epsilon amino groups are detected and the SB does not interfere. Collagen crosslinks do not interfere in this method but glycoproteins with acid labile glycosidic bonds and free sugars have been shown to interfere (42).

1.8.2: Periodate Oxidation/DDL Assay

Periodate oxidation of a glycated protein causes release of the sugar group from protein and production of performic acid and formaldehyde. The bonds cleaved by periodate oxidation (based on Malaprade's rule) are;



Formaldehyde is released only from C-1 and C-6 atoms of straight chain hexoses which are adjacent to a C-OH or C=O group (43). AP in the pyranosyl or furanosyl form will therefore release one mole of formaldehyde

per mole of AP whereas AP in the acyclic form will release two moles of formaldehyde per mole AP (fig 1.10). SB in the acyclic form will also release one mole of formaldehyde but can be removed by dialysis and since virtually all the AP exists in the ring form, approximately one mole of formaldehyde is released per mole of AP. The released formaldehyde is detected by conversion to diacetyldihydrolutidine (DDL) as in the Hantzsch reaction (44). Free sugars interfere in this method and have to be removed by dialysis and so do glycoproteins with chain terminal reducing sugars or sialic acid residues. However, this method has the advantage that it is stoichiometric and will detect glycation at both alpha and epsilon amino groups.

1.8.3: Phenylhydrazine Assay

Amadori products possess carbonyl groups and methods which measure these could be used to quantify glycation. Earlier attempts to measure glycation of Hb using phenylhydrazine were unsuccessful and this was attributed to the small percentage of glucose-AP existing in the acyclic form (156). More recently, the method has been used to assay for glycation of albumin (157) and reaction between glyceraldehyde and Hb (158). Phenylhydrazine (via its amino group) reacts with carbonyl from AP to form a coloured hydrazone whose absorbance is measured at 405nm (fig 1.11).

1.8.4: Fructosamine Assay

This assay is used in routine clinical laboratories to measure the AP (fructosamine) in serum protein. In alkaline conditions, ketones (like the AP) can be reduced to give an eneaminol, which undergoes dehydration to produce a strong reducing agent or enediol. This enediol can reduce nitroblue tetrazolium (NBT) by an unknown mechanism to produce a blue black colour which can be quantified (45). Ketoses enolize faster than aldoses and so the SB and free glucose will not interfere in this method. A

major problem with the fructosamine assay is that it is difficult to standardize because the colour varies with protein, dye concentration, pH, temperature, and time (46, 47).

1.8.5: Borohydride Reduction

Reduction by borohydride occurs at the C=N bond in SB and C=O in glucose-derived AP stabilizing the sugar-protein link to form deoxyaminosorbitol (ie glucitol-lysine) as the major product whereas a small amount of deoxyaminomannitol is also formed from the AP. To increase sensitivity, tritiated borohydride is used (48) and following reduction the protein is hydrolysed to its constituent amino acids. The glucitol-lysine can be separated by amino acid analysis since it elutes between the aromatic and basic amino acids. This method has the advantage of being very sensitive, but is not very specific and free sugars, SB, collagen crosslinks and glycoproteins will interfere.

1.8.6: Phenylboronate Affinity Chromatography

An affinity chromatography method for measuring glycated proteins was developed in 1980 using boronic acid linked to agarose by an m-aminophenyl spacer (49). At pH >7, the boronic acid ionises so that the OH groups can be replaced by cis diol groups from the AP. The labile SB dissociates during chromatography, and glycoproteins do not bind as strongly as glycated proteins. Bound protein is eluted by washing with a buffer containing a high concentration of sorbitol or HCl. The amount of glycated albumin bound to the column varies from 6.3% (50) to 20% (51) probably due to differences in conditions such as temperature, pH, sample size, flow rate and buffer composition. This method has been adapted for routine use in the measurement of glycated Hb by Gould et al (52).

1.8.7: Furosine Assay

Acid hydrolysis of glycated proteins will result in the AP being converted to;

1: furosine (30%)

2: lysine (50%)

2: pyridosine (20%)

Furosine can be separated by HPLC and its A_{280} measured (42). This method is very sensitive ie only requires 15-25ug of protein, is very specific, and free sugars, SB, glycoproteins and collagen crosslinks do not interfere. However, yields of furosine are poor ie only 30%, and only lysine glycation is detected.

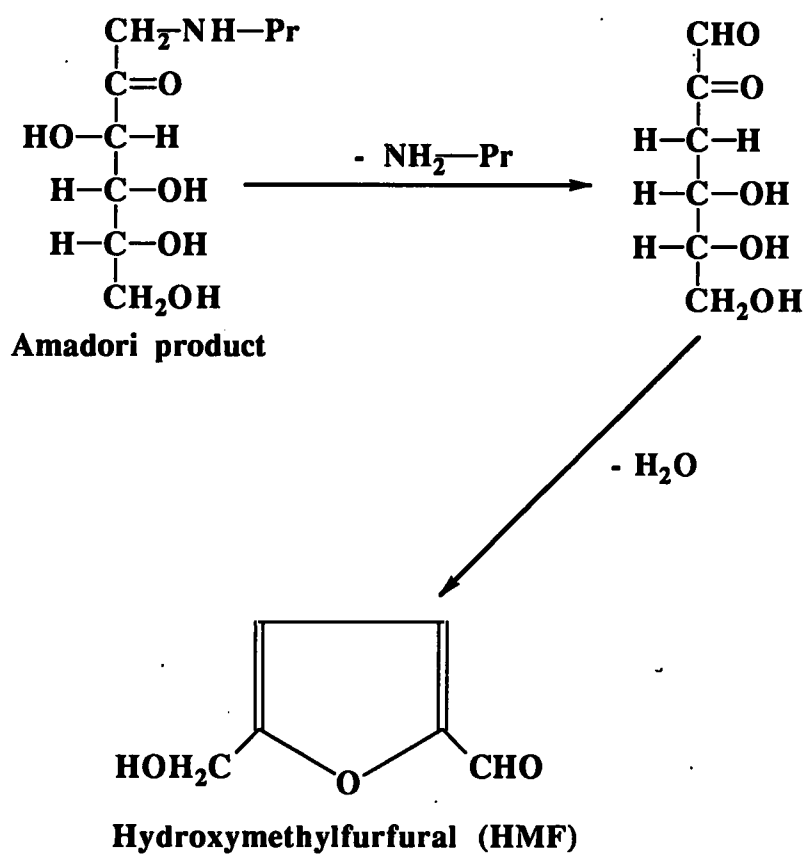


Fig 1.9: Formation of Hydroxymethylfurfural (HMF)

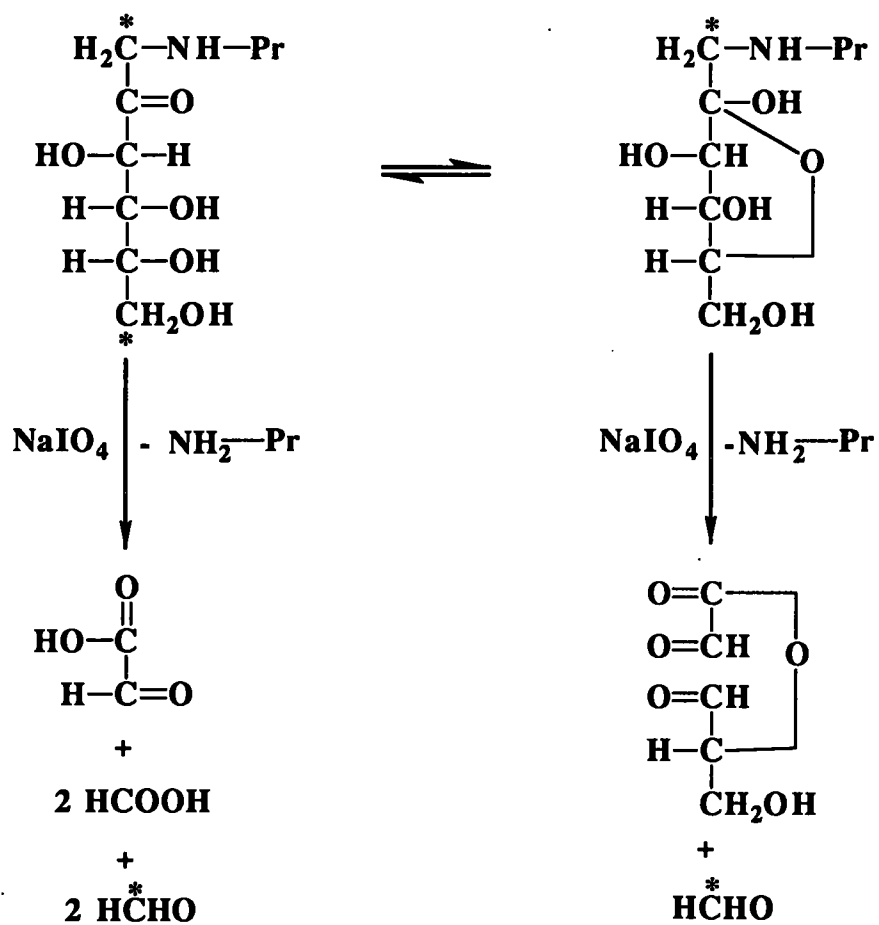


Fig 1.10: Periodate oxidation of glucose-AP (asterix denotes C atom liberated as HCHO).

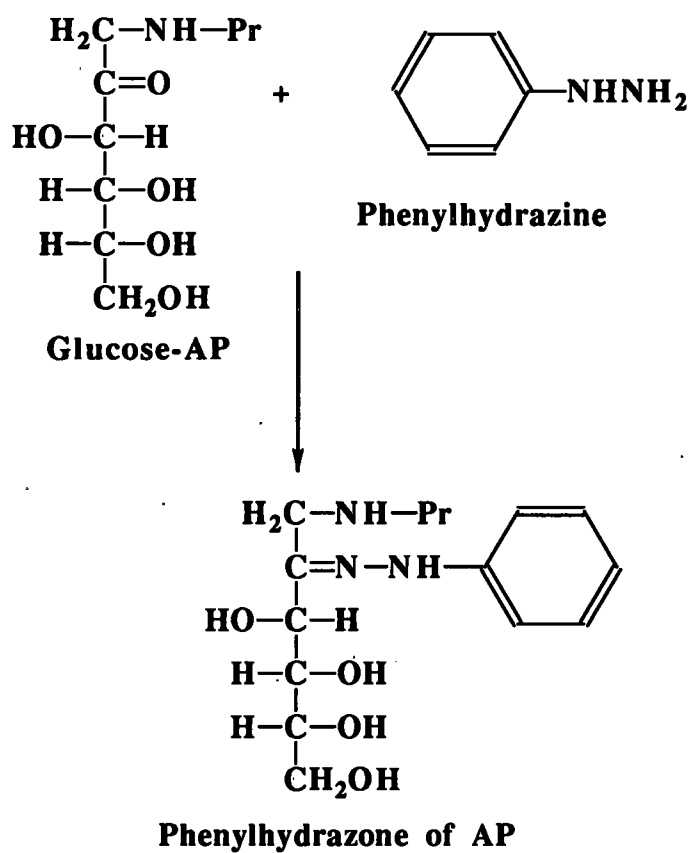


Fig 1.11: Formation of AP phenylhydrazone

1.9: Measurement of AGE

At present there are no simple specific assays for measuring AGE and those for measuring pyrraline (24) and pentosidine (25) are restricted to a few laboratories. The simplest methods for AGE detection rely on their characteristic fluorescence and crosslinking properties.

1.9.1: Fluorescent-AGE

Many AGE have characteristic absorbance and fluorescence spectra which can be used for their detection. This is a very crude method since a large number of other compounds can also fluoresce. For the results to be meaningful, values have to be normalised with respect to protein concentration.

1.9.2: Crosslinked-AGE

Polymerisation of molecules as a result of AGE formation has been demonstrated for many proteins including lens crystallin (53), albumin (54), RNase (55) and collagen (56), and this property can be used to detect AGE. First the protein is treated with mercaptoethanol or dithiothreitol to eliminate crosslinks due to disulphide bonds, subjected to SDS-PAGE and then scanned by laser densitometry to calculate the percentage oligomer (57).

1.10: Role of Glycation and AGE in Diabetes and Ageing

One of the major problems that individuals with long standing diabetes face is the increased susceptibility to secondary complications which include;

- 1: retinopathy
- 2: cataract
- 3: atherosclerosis
- 4: nephropathy
- 5: neuropathy

6: limited joint mobility

As a consequence of these complications, the life expectancy of diabetics is only 2/3 of that of the general population (58). Hyperglycaemia is a major causative factor in the development of these complications and diabetics with poor blood glucose control are particularly at risk (59). Furthermore, the incidence of many of these complications increases with age even in non-diabetics.

Glycation of several proteins has been shown to increase in diabetes (60, 61), and virtually any protein can be glycated in vitro. Although earlier work has suggested that protein glycation increases with age (62, 63), more recent studies have not confirmed these findings (64, 65). Glycation would not be expected to increase with age since it should reach steady state levels in equilibrium with glucose concentrations, AGE formation and protein turnover.

Theoretically, glycation and AGE formation could alter protein conformation and may impair function in several ways;

- 1: altered enzymatic activity**
- 2: decreased ligand affinity**
- 3: modified protein half life**
- 4: blocking of proteolytic sites**
- 5: altered immunogenicity**

Glycation of RNase in vitro reduces its activity by 50% probably by blocking lysine-41 essential for its function (55). In contrast, glycation increases the activity of aldose reductase in vitro (66), but has no effect on the proteases trypsin and chymotrypsin (67). In vitro glycated insulin has reduced biological activity which can be demonstrated on fat cells as reduced

glucose oxidation, increased fatty acid synthesis and reduced antilipolytic properties (68). Because of its very short half life, glycation of insulin in vivo would not be expected, and so the above findings are only of theoretical interest.

About 6-15% of albumin, the major serum protein is glycated in normoglycaemic individuals (69), but a two fold rise occurs in diabetics (70). The major site of glycation in albumin is lysine-525 in vivo (71) and glycation of the same site occurs preferentially in vitro causing a conformational change (72). As a result, the binding of bilirubin and fatty acids decreases by 2 and 20 fold respectively. However, binding of palmitate is unaffected by glycation of albumin to extents found in vivo (73).

Glycation of LDL is increased in diabetics where upto 5% of lysine residues in apoprotein B are modified compared to only 3% in non-diabetics (74), and this extent of glycation has been reported to be sufficient to impair its binding and uptake via the LDL receptor in fibroblasts and endothelial cells in vitro (75). Since 75% of LDL clearance is mediated by the LDL receptor, this may account for the higher plasma levels of LDL in diabetics (76). Schleicher et al however, have found that glycation of LDL to extents seen in diabetes does not affect its catabolism by fibroblasts (77). Formation of AGE on LDL increases their binding and uptake by macrophages which possess a specific receptor that recognises AGE but not the AP in vitro (78). This pathway is independent of the classical scavenger pathway and the receptor has recently been purified and shown to be a protein of 90KD (79).

Glycation of lens protein in vitro and in vivo was first reported by Stevens et al in 1978 who suggested that conformational changes induced by glycation could expose SH groups to oxidation and disulphide formation (80). Furthermore, lens protein incubated with glucose or glucose-6-phosphate slowly becomes brown and produces fluorescence spectra

similar to that by lens protein from cataract (81). Increased AGE in addition to disulphide crosslinks may therefore be involved in cataract formation.

Collagen, the major structural protein has increased glycation in diabetes for virtually every tissue analysed including basement membranes from both kidneys and lens capsule (82), aorta (83), tail tendon (84) and skin (85). Earlier work has reported increased glycation of collagen with age from skin (86), glomerular basement membrane (87), bone, tendon and cartilage (88). However, recent studies using more specific methods have found no increase in glycation with age (89) or only slight increases that can be correlated with age associated increases in glycaemia (90). The above discrepancies are probably due to differences in the methods used to assay for glycation, since most of the earlier work is based on the TBA assay which is susceptible to interference by glycoproteins whereas the more recent methods ie furosine method have overcome these problems. Increased crosslinking, rigidity, browning, fluorescence and reduced susceptibility to proteolysis occurs with collagen in vitro (91). Such changes have been used as markers for AGE and have been shown to increase with age (92) and in diabetes (93). Whether the above changes are due solely to glycation-induced AGE is not yet certain since other processes such as free radicals may also have the same effect (94). AGE crosslinks in collagen have been implicated in the limited joint mobility (LJM) syndrome seen in 40% of insulin-dependent diabetics. This suggestion has been supported by the finding that collagen from patients with LJM has higher fluorescence and crosslinking compared to those without (95). Brownlee et al using immobilised collagen on agarose have shown that collagen-AGE can covalently trap LDL. At a constant LDL concentration, covalent trapping increased linearly with the extent of collagen-AGE (96). Excessive trapping of plasma proteins by hyperglycaemia-induced collagen-AGE in the arteries

and glomerular basement membrane may contribute towards the accelerated development of atheroma and nephropathy respectively in diabetes.

AGE formed on glycated myelins either in vitro or from diabetics increases their susceptibility to phagocytosis by macrophages stimulating them to secrete proteases (97). Whether this contributes towards the demyelination of nerve fibres seen in diabetic neuropathy has not yet been proven.

1.11: Fructation and the Sorbitol Pathway

Since glucose is the major metabolic sugar, glucation has received the most attention. However, other reducing sugars present in vivo can also participate in glycation and do so much faster than glucose in vitro. In vivo, these sugars are present in minute concentrations and have therefore been ignored except for galactose which has been investigated with respect to galactosaemia (98). In vitro studies using radiolabelled sugars have shown that fructose reacts and forms the labile SB some 8 times faster than glucose with Hb (11). In addition, fructose induces AGE formation upto 10 times faster than glucose in vitro with albumin (99), RNase (57), lens protein (100) and collagen (101).

Rich sources of fructose in the diet include fruits, honey and sucrose. Fructose is considered safe for diabetics and is a permitted sweetener in diabetic foods. A number of reasons have been given for this;

- 1: Since fructose is much sweeter than glucose, one is likely to consume less.
- 2: Fructose is rapidly metabolised in the liver and its levels in the plasma are well below 1mM.
- 3: Fructose unlike glucose does not require insulin for its metabolism and can enter glycolysis.

In the liver, fructose can be converted to glycogen which will ultimately release glucose and for this reason, some diabetologists have argued against using fructose in diabetic foods. The relationship between fructose and other metabolic pathways is shown in fig 1.12. The possibility that dietary fructose may contribute towards in vivo glycation has been virtually ignored presumably because of its low plasma levels (102). However, there are interesting studies regarding dietary fructose in diabetes;

- 1: A study in Japan has shown that diabetics have higher serum fructose levels compared to non-diabetics after ingestion of a fructose-rich diet (103).
- 2: A case study of a diabetic with high glycated Hb values but normal blood glucose levels highlights the dangers of eating too much fructose (104). This individual was on a high fructose diet and tests for reducing sugars in urine revealed the presence of fructose. When the high fructose diet was stopped, glycated Hb values returned to normal within two months yet daily blood glucose levels remained the same.
- 3: Patients with hereditary fructose intolerance have elevated serum fructose levels and have higher than normal levels of glycated Hb (105).
- 4: Long term administration of fructose in the diet has led to lesions similar to diabetic microangiopathy in rats (106).

Apart from dietary fructose, there are regions in the body where the fructose accumulates and in these regions the sorbitol pathway operates. In diabetes the sorbitol pathway is highly active and glucose is converted to sorbitol and fructose under the influence of the enzyme aldose reductase (107). The sorbitol pathway has been detected in the lens (108), nerves (109), arteries (110) and retina (111) ie regions associated with diabetic

complications. Furthermore, the sorbitol pathway is also of interest in diabetes because it is believed that sorbitol accumulation within cells can produce a high intracellular osmotic pressure which may cause cell damage and thus contribute towards the pathogenesis of diabetic complications. However, very little attention has been paid to the levels of fructose which can increase by 23 fold reaching and even exceeding the levels of glucose (112). One study has reported concentrations of fructose upto 12mM in the lens (108), and clearly significant fructation may occur here. Indirect evidence for in vivo fructation by the sorbitol pathway has been presented by Suarez et al who demonstrated that administration of sorbinil, an aldose reductase inhibitor decreased collagen-linked fluorescence in diabetic rats (113). In contrast, Cohen et al found no decrease in collagen-linked fluorescence after administration of sorbinil (114). In both these studies, fluorescence of an extracellular protein was measured, whereas the sorbitol pathway is an intracellular event. Sorbinil does not affect levels of glycaemia (115), but one report has claimed it to be a free radical scavenger (80) and therefore its effect may be by an alternative mechanism. Odetti et al have also reported decreased collagen-linked fluorescence in diabetic rats receiving rutin which is also an aldose reductase inhibitor (116). It is noteworthy that glycation of aldose reductase actually increases its activity in vitro (66). Finally, direct evidence for in vivo fructation has been presented by McPherson et al who have reported that 20% of the AP in lens protein is derived from fructose (57).

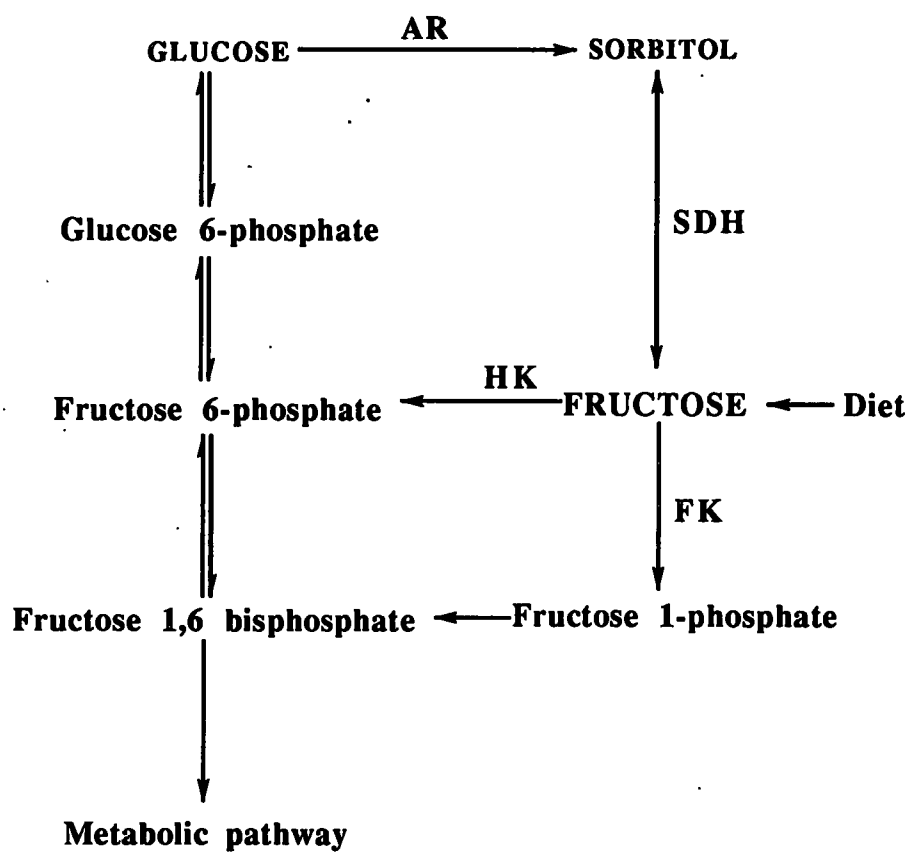


Fig 1.12: Metabolism of fructose (key; aldose reductase (AR), fructokinase (FK), hexokinase (HK), sorbitol dehydrogenase (SDH)).

1.12: Inhibitors of Glycation and AGE Formation

There is a possibility that the body may possess mechanisms to protect against glycation and AGE formation. An enzyme alpha-ketoglutaraldehyde dehydrogenase found in the liver can inactivate deoxyglucosones in vitro (117), and macrophages can recognise and endocytose molecules modified by sugars (78). Preliminary studies by Vlassara et al have shown that the ability of mouse peritoneal macrophages to remove AGE-proteins decreases with age (118). Such an effect in humans may explain the higher incidence of diseases such as atherosclerosis with increasing age. A variety of amines in plasma such as taurine, free amino acids, creatine and guanidine are capable of blocking carbonyl groups on sugars, AP's and 3-DG and may reduce sugar-induced damage in vivo. However, the efficiency of these natural defence mechanisms in vivo is not yet known.

At present there is considerable interest in pharmacological compounds which could reduce or prevent glycation and post-Amadori reactions in vivo because of their therapeutic potential.

1.12.1: Aminoguanidine

This nucleophilic hydrazine with three amino groups has received the most attention. Brownlee et al in 1986 were the first to demonstrate that aminoguanidine could reduce glucose-derived fluorescence and crosslinking of collagen and albumin in vitro (119). In the same study, aminoguanidine administered to diabetic rats for 16 weeks reduced crosslinking of aortic collagen and basement membrane thickening in the kidneys. A more recent study by Odetti et al has shown that aminoguanidine not only reduces collagen-linked fluorescence in diabetic rats but has no effect on glycaemia or glycated Hb levels (116). Brownlee et al proposed that aminoguanidine blocked free carbonyl groups on AP therefore inhibiting AGE formation but not glycation (120). The protein-

aminoguanidine complex which would be expected to form as a result has never been identified and more recent studies suggest that there may be more than one site of action. Indeed, aminoguanidine can reduce glycation in vitro by reacting with the free sugar (121), and AGE formation by blocking 3-DG (122). The possibility that aminoguanidine may reduce oxidative processes since it is a known inhibitor of diamine oxidase have not yet been excluded (123). At present nothing is known about the metabolism of aminoguanidine or its complexes.

In vivo aminoguanidine could react with other naturally occurring carbonyl compound and may impair their function for eg reaction between aminoguanidine and pyridoxal phosphate may lead to vitamin B₆ deficiency (124).

1.12.2: Acetylsalicylic acid

A study by Cotlier in 1981 suggested that frequent use of aspirin may protect against diabetic cataract. This study found that the prevalence of cataract was significantly lower in diabetics with rheumatoid arthritis receiving high doses of aspirin compared to a matched population on no aspirin (125). Acetylation of amino groups by aspirin (acetylsalicylic acid) has been shown to reduce glycation of albumin, haemoglobin (126) and lens protein (127) in a dose dependant manner in vitro, and this led to the suggestion that aspirin may protect against diabetic cataract by preventing glycation (128). However, other analgesics such as paracetamol and ibuprofen also have a protective effect against cataract yet cannot acetylate proteins (129). Therefore, the protective effect of aspirin may be due to other mechanisms since aspirin has been reported to reduce glycaemia (130), inhibit sorbitol pathway activity (125) and stimulate the release of insulin (131). A recent study has shown that administration of aspirin in diabetic rats reduced glycation of lens protein without affecting glycaemia (132).

1.12.3: Antioxidants and Free Radical Scavengers

Vitamin E has been shown to decrease glycation of albumin in a dose dependant manner in vitro (133), and more recently therapeutic doses of the vitamin (800mg/day) have been shown to reduce HbA_{1c} levels in diabetics (134). The mechanism of action of vitamin E is not yet known but could reduce autoxidation of sugars preventing their incorporation into proteins. Another antioxidant, vitamin C has also been shown to inhibit glycation of serum proteins both in vitro and in vivo (135). Free radical scavengers may prevent fragmentation, fluorescence and crosslinking of proteins by sugar-derived free radicals. The free radical scavenger sorbitol has been reported to inhibit glucose-derived fragmentation of albumin in vitro (33).

1.13: Brief Review of Proteins Used in this Study

The model proteins used in this study include serum albumin (human and bovine), lysozyme and ribonuclease. These proteins were chosen since they are soluble, relatively inexpensive and have already been studied with respect to glycation.

1.13.1: Albumin

Albumin is the major plasma protein with a half life of 19 days and a concentration of approximately 40mg/ml in vivo. The essential function of albumin is to maintain osmotic pressure of blood and to transport a variety of substances such as fatty acids and bilirubin. Albumins have a high content of lysine, arginine, cysteine and aspartic acid and a low content of tryptophan and methionine. Due to the high cysteine content albumin has 17 disulphide bonds which give the protein its stability. Both human serum albumin (HSA) and bovine serum albumin (BSA) contain 59 lysine residues per molecule. Albumin can bind a number of positively charged ligands

including Ca^{2+} , Cu^{2+} , Ni^{2+} and Zn^{2+} as well as drugs such as acetylsalicylic acid, warfarin and ampicillin (reviewed in (136)).

1.13.2: Lysozyme

This enzyme can lyse bacterial walls and egg white is a rich source of the protein. Lysozyme is a single polypeptide consisting of 129 amino acids and has 4 disulphide bonds which give the molecule its stability. Lysozyme contains 6 lysine and 11 arginine residues and has a molecular weight of 14,300. At present there is no information on in vivo glycation of lysozyme. However the protein has been glycated in vitro and readily polymerises (137).

1.13.3: Ribonuclease

This enzyme can cleave ribonucleic acids (RNA) and has a molecular weight of 13,700 with 4 disulphide bonds. The molecule is a single polypeptide of 124 amino acids with 10 lysine and 4 arginine residues. At present there is no evidence for in vivo glycation of RNase, however, in vitro studies have shown that Lys-1 and Lys-41 are more reactive than the other lysine residues (9). Glycation at Lys-41 in RNase causes a loss in activity of the enzyme (55).

1.14: Aims and Objectives

The specific aims for each experimental chapter are listed after the introduction to that chapter. Here the overall aims of the thesis will be summarised and listed below;

- 1: To improve sensitivity, speed and yield of DDL in the periodate method so that it can be used as a rapid assay to measure absolute values of glycation in small amounts of intact protein.

- 2: To improve sensitivity, speed and yield of HMF in the TBA method so that it can be used as a rapid assay for small amounts of glycated collagen.
- 3: To evaluate the potential role of fructose during in vivo glycation and AGE formation by in vitro experiments using model proteins.
- 4: To investigate whether other glycation assays apart from the periodate method will underestimate the true AP levels in fructated BSA and the possible reasons for this.
- 5: To elucidate mechanisms of AP and AGE formation using different proteins, phosphate concentration, pH, sugars and inhibitors.
- 6: To evaluate the possible role of autoxidative glycation and free radical mediated protein fragmentation in vivo by studying the reaction in vitro.

CHAPTER 2

Materials and Methods

2.1: In Vitro Glycation Procedure

Proteins of known concentration were incubated in different sugars (M/l) in either sodium or potassium phosphate buffer (M/l), pH 7.4 at 37°C in a Memmert universal oven (Model No UM 100/l) for a stated period of time. Where necessary, 0.05M Tris or HEPES, pH 7.4 was used instead of phosphate buffer. To prevent bacterial growth, solutions contained 3mM sodium azide or were passed through polydisc (0.45u) (Whatman, Maidstone, Kent, UK) under sterile conditions. Incubations with azide have the disadvantage in that azide may interfere in reactions of glycation and AGE formation for eg azide could act as a free radical scavenger. Incubations performed under sterile conditions overcome these problems but are unsuitable for prolonged incubations since bacterial growth may occur. Furthermore facilities must be available to perform sterile techniques. The pH of incubations was checked periodically using a Schott Gerate pH meter (Model No CG 727) and adjusted to the desired value if necessary. All manipulations with proteins were performed on ice. At the appropriate time, glycation was terminated by freezing the sample to -40°C.

2.2: Removal of Unbound Sugar

Dialysis tubing with a mwco of 12-14,000 (Medicell International Ltd, London, U.K) was used for albumin and that for lysozyme and RNase, had a mwco of 6-8,000 (Spectrum Medical Industries, Los Angeles, CA, USA).

Preparation of Dialysis Tubing: Tubing was soaked for one hour in 1% acetic acid followed by rinsing in distilled water for 15 mins. The tubing was then soaked for 15 mins in a solution of 1% sodium carbonate containing 10mM EDTA. The tubing was heated to 75°C twice in this solution before

being rinsed in distilled water and heated to 75°C again. After a quick rinse, the tubing was stored at 4°C in distilled water to which a few drops of chloroform had been added.

Dialysis Procedure: Samples were dialysed by stirring in 2l of distilled water or buffer at 4°C in the dark over a period of 3-4 days with 8 changes. A minimum of 4 hours was allowed between each change. Adequate dialysis was important for removal of free sugars which can interfere in protein as well as glycation assays. Extensive dialysis has also been reported to remove the labile SB which can interfere in the periodate assay (57). Dialysed proteins were stored frozen at -40°C, and this has been reported to cause no increase in glycation (138).

2.3: Collagen Digestion

Dialysed glycated collagen was centrifuged at 9000g for 10 mins and the precipitate resuspended in 0.05M sodium phosphate buffer, pH 7.4 containing 200IU collagenase and incubated at 37°C for 48 hours. Chloroform and toluene (5ul each) were added to prevent bacterial growth. After centrifugation at 9000g for 10 mins, the supernatant was collected and stored frozen.

2.4: Protein Assays

Protein concentrations were determined according to three different methods;

2.4.1: Lowry Protein Assay

In the method of Lowry et al (139), reagent A (2% Na₂CO₃ in 0.1M NaOH) and reagent B (0.5% CuSO₄.5H₂O in 1% Na-K tartarate) was mixed in the ratio of 50:1 respectively, and 1ml of this was added to 0.1ml of sample or standard followed by incubation at room temp for 30 mins. After incubation,

0.1ml of Folin Ciocalteau's phenol reagent was added whilst vortexing tubes, which were then left at room temp for 15 mins before measuring the absorbance at 750nm using a Gilford 260 Spectrophotometer. A calibration graph ranging from 0-50ug BSA was also prepared (fig 2.1). All samples were assayed in duplicate and the results expressed as nmols protein/ml or mg/ml.

2.4.2: Pierce BCA Microassay

A microversion of the bicinochoninic acid (BCA) method of Smith et al (140) was used. To 0.02ml of sample or standard in microplate was added 0.2ml of reagent A (Na_2CO_3 , NaHCO_3 , BCA detection reagent and sodium-tartarate in 0.2 M NaOH) and reagent B (4% CuSO_4) in the ratio of 50:1. The microplate was covered and stirred in a Wellmixx 1 microplate stirrer (Model No WM 503) for 1 min before incubation at 37°C for 30 mins. Absorbance at 570nm was measured using a Biotek microplate reader (Model No EL311) with a 570nm filter. A calibration graph using known amounts (0-20ug) of the protein being assayed was also prepared. The calibration graph for 0-20ug of BSA is shown in fig 2.2. All assays were performed in duplicate and the results expressed as nmols protein/ml or mg/ml.

2.5: Reductive Methylation of Glycated BSA

Reductive methylation of BSA was performed according to the procedure of Jentoft and Dearborn (142). Glycated BSA (~5mg/ml) was stirred slowly in 0.05M sodium phosphate buffer, pH 7.4 with 2mM formaldehyde and 20mM sodium cyanoborohydride for 6 hours at 4°C in the dark and then dialysed overnight against 2l of the buffer. It was necessary to repeat this procedure (5-6 times) until adequate blocking of amino groups was achieved as measured by the TNBS method. Finally the methylated BSA was dialysed

exhaustively to ensure complete removal of formaldehyde and sodium cyanoborohydride. Removal of formaldehyde was particularly important since it can interfere in the periodate assay used to quantify glycation.

2.6: Proteinase K Digestion

Prior to any fluorescence readings, the reductively methylated (RM) BSA was digested by 12.5 ug/ml of proteinase K for 18 hours in 0.05M sodium phosphate buffer, pH 7.4 at 37°C and boiled for 1 min to destroy enzyme and then briefly centrifuged. Activity of the enzyme was measured by the rise in amino groups as detected by the TNBS method.

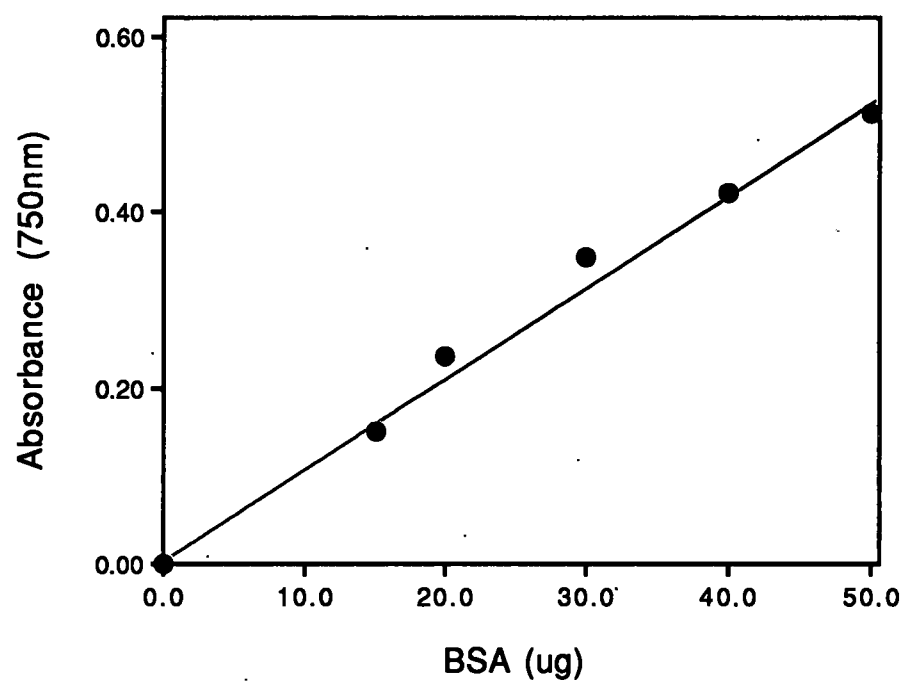


Fig 2.1: Calibration graph for BSA standards (0-50ug) by Lowry protein assay

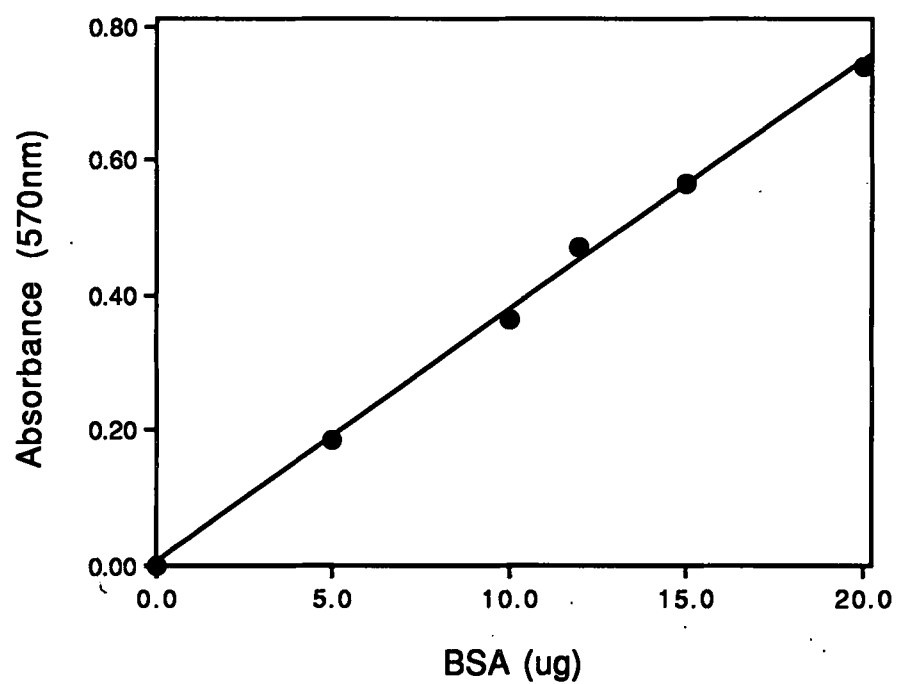


Fig 2.2: Calibration graph for BSA (0-20ug) by the Pierce BCA microassay

2.7: Trinitrobenzene Sulphonic Acid (TNBS) Method

Amino groups were determined according to the method of Spadaro et al using TNBS (143). To 0.4ml of sample or standard was added 0.2ml of 5mM TNBS followed by 0.4ml of 1M potassium borate buffer, pH 9.2. After incubation at room temp for 40 mins, 0.2ml of 18mM Na₂SO₃ in 2M NaH₂PO₄ was added and the tubes vortexed. Absorbance was measured on a Gilford 260 Spectrophotometer. A calibration graph was prepared using known amounts (0-62.5nmols) of N-acetyl-L-lysine-N-methylamide (NALMA) and is shown in fig 2.3. All assays were performed in duplicate and the results expressed as nmols NH₂/ml of protein solution.

2.8: Measurement of Glycation

Assays used to measure glycation in this project provided a measure of the AP alone since the SB was removed during dialysis. These assays include;

2.8.1: Periodate Oxidation Assay (Gallop procedure)

The procedure of Gallop et al was used (43). The sample or standard was made upto a volume of 700ul in distilled water and 20ul of 1M HCl followed by 100ul of 0.1M NaIO₄ was added before vortexing. Samples were left at room temp for 30 mins to allow oxidation to occur before cooling in ice for 10 mins. Ice cold 10% ZnSO₄ (300ul) was added followed by 100ul of 1.4M NaOH and the tubes centrifuged for 10 mins at 1000g using a Heraeus Biofuge A bench-top centrifuge. An aliquot of the supernatant (700ul) was removed and 1ml of formaldehyde detection reagent (2M ammonium acetate, 0.02M acetylacetone) was added to this. All tubes were tightly sealed and incubated at 37°C for 60 mins in a Griffin 100 Series waterbath. The samples were incubated at room temp for 10 mins before reading the fluorescence emission at 510nm after excitation at 410nm (slit width=10nm, fix scale=1, photomultiplier gain=1 and sensitivity=3) using a LS5-B Perkin-

Elmer Luminescence Spectrometer. In some cases the absorbance at 412nm was measured using a PYE Unicam SP6 Spectrophotometer. A calibration graph for fructose standards (0-50nmols) was also prepared as shown in fig 2.4. All assays were performed in duplicate and the results expressed as moles AP/mole protein.

2.8.2: Periodate Oxidation/DDL Microassay

A microversion of the periodate method of Gallop et al (43) was developed in this project. The volume of sample or standard was adjusted to 40ul with water and incubated with 20ul of 0.1M HCl and 20ul of 0.05M sodium periodate at room temp for 30 mins. To terminate oxidation, samples were cooled in ice for 10 mins and mixed with 20ul of 15% ZnSO₄ and 20ul of 0.7M NaOH. Both reagents were precooled and samples were vortexed throughout addition. They were then centrifuged for 10 mins at 9,000g to remove precipitated ZnIO₄, and 100ul aliquots of the supernatant fluid were transferred to microplate wells. Formaldehyde detection reagent (FDR) was freshly prepared by mixing 46ul of acetylacetone in 10ml of 3.3M ammonium acetate; 200ul of this reagent was added to each well and mixed thoroughly with sample using the microplate mixer and diacetyldihydrolutidine (DDL) allowed to develop by incubating for 1 hour at 37°C. Reagent blanks (100ul water + 200ul FDR) were incubated at the same time. The microplate was inserted into the microplate reader and absorbance of reagent blanks at 405nm subtracted automatically from absorbance of sample solutions. A calibration graph using 0-50nmols fructose was also prepared (fig 2.5). All assays were performed in duplicate and the results were expressed as moles AP/mole protein.

2.8.3: Thiobarbituric Acid (TBA) Microassay

A microversion of the TBA method of Parker et al was used (144). To 100ul of sample or of standard in bijoux bottles was added 100ul of 0.5M oxalic acid. The bottles were tightly sealed and incubated in a Tefal pressure cooker at 124°C and 124 KPa. After allowing the samples to cool for 15 mins at room temp, they were transferred to eppendorf tubes followed by addition of 100ul of 40% trichloroacetic acid (TCA). The samples were centrifuged at 9,000g for 10 mins and 200ul aliquots of the supernatant were transferred to microplate wells. To these aliquots, 67ul of 0.05M TBA (which has been adjusted to pH 6 with NaOH) was added to each well and mixed thoroughly. Microplates were incubated at 37°C for 30 mins to allow chromophore to develop and allowed to cool to room temp for a further 15 mins. Standards of 0-50nmols fructose were prepared simultaneously with each assay (fig 2.6). Absorbance at 450nm was measured using the microplate reader. All assays were performed in duplicate and the results expressed as nmols HMF/mg protein.

2.8.4: Dinitrophenylhydrazine (DNPH) Microassay

A modified version of Fields and Dixon was used (145). To 0.5ml of protein or standard ketone solution made in distilled water was added 0.24ml of 10M HCl, followed by 0.1ml of 5mM dinitrophenylhydrazine prepared in 2M HCl. The tubes were vortexed and left at room temp for 30 mins before transferring 0.3ml aliquots to microplate wells. Absorbance at 405nm was measured in the microplate reader. A calibration graph was prepared using 0-50nmol ketoglutarate standards (fig 2.7). All assays were performed in duplicate and the results expressed as moles carbonyl/mole protein.

2.8.5: Phenylboronate Affinity Chromatography

This method was adapted from that of Middle et al (146). A 200ul sample containing 2mg albumin in washing buffer (0.25M ammonium acetate, pH 8.5 containing 0.05M magnesium acetate and 3mM sodium azide) was applied to a 1 X 2cm column packed with Matrex PBA-10 resin (Amicon Corp, Danvers, MA, USA). After washing to remove unadsorbed protein, glycated albumin was eluted in washing buffer containing 0.25M sorbitol. Flow rate was maintained at 25ml/hour using a LKB 2120 Varioperpex II pump and the A₂₇₉ was monitored by a LKB 2138 Uvicord S connected to a LKB 2065 Chopper-bar recorder. The column was regenerated in 0.1M acetic acid. The results were expressed as percentage glycation.

2.8.6: Serum Fructosamine Assay

This assay was kindly performed by Dr Martin Hartogg, Department of Medicine, Southmead General Hospital, Bristol, UK and is according to the procedure of Johnson et al (45).

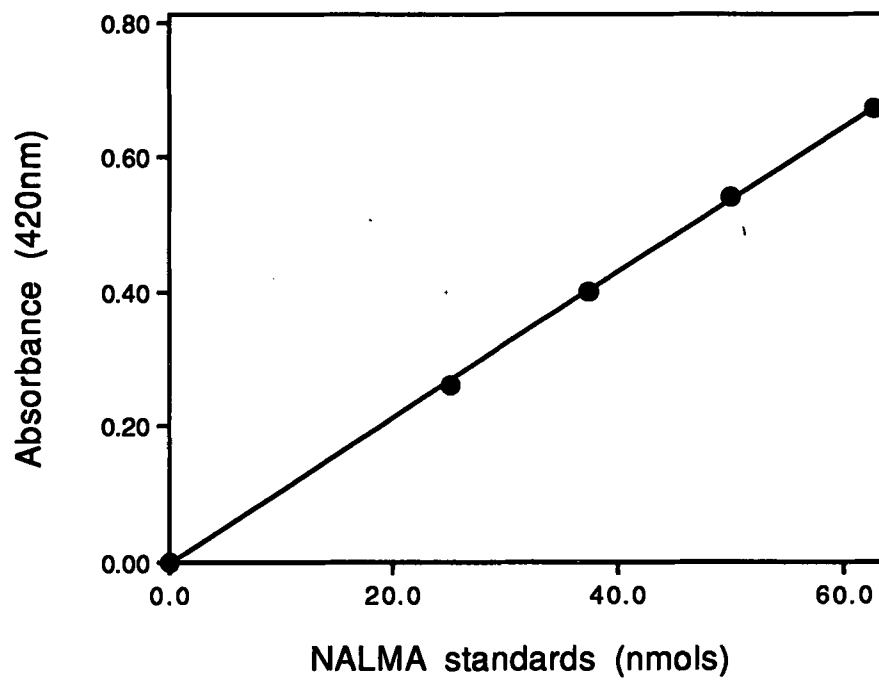


Fig 2.3: Calibration graph for NALMA (0-62.5nmols) by TNBS Assay

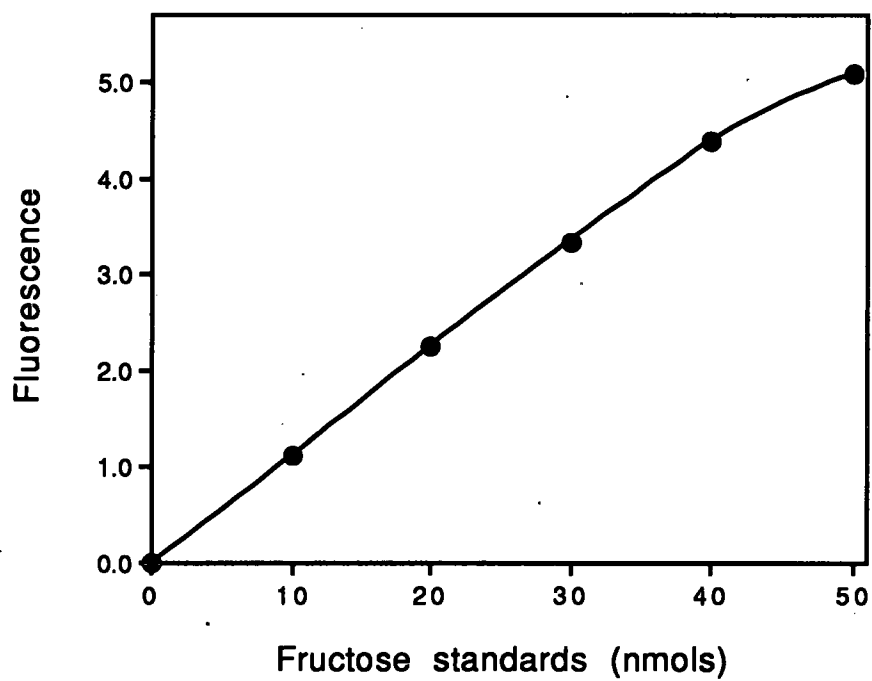


Fig 2.4: Calibration graph for fructose standards (0-50nmols) by the periodate assay (Gallop procedure)

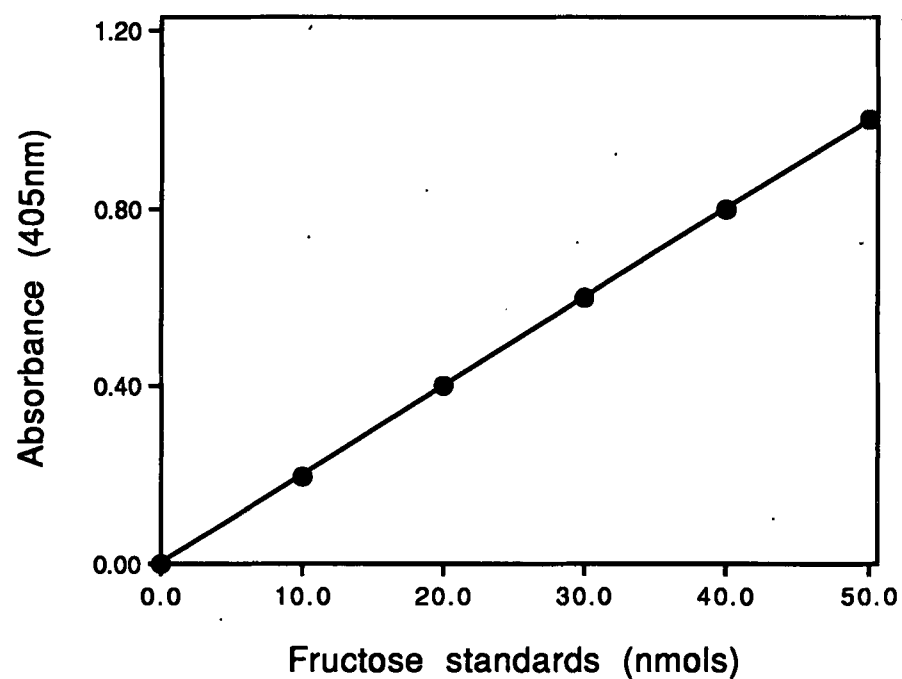


Fig 2.5: Calibration graph for fructose (0-50nmols) by the periodate microassay

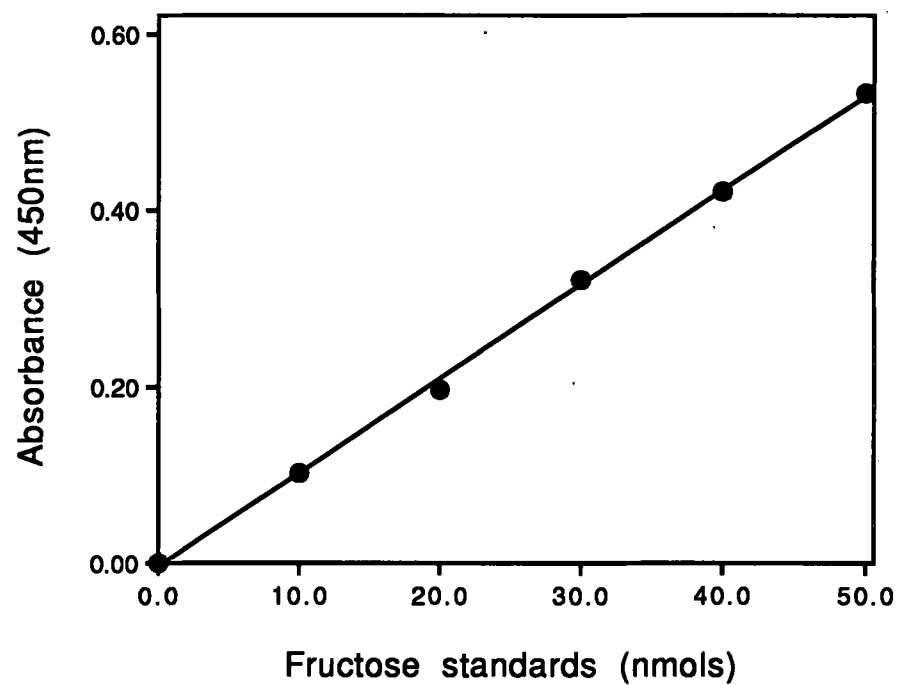


Fig 2.6: Calibration graph for fructose (0-50nmols) by the TBA microassay

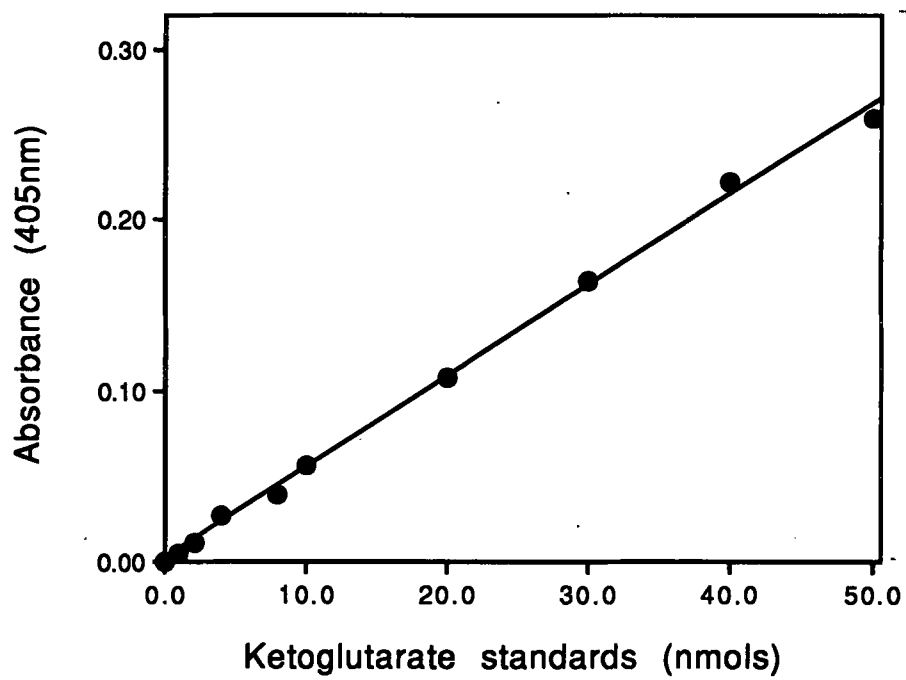


Fig 2.7: Calibration graph for ketoglutarate (0-50nmols) by the DNPH microassay

2.9: Measurement of AGE

Sugar-induced AGE on proteins can be fluorescent and / or crosslinked and these properties were used to detect them;

2.9.1: Fluorescent-AGE

The procedure of Suarez et al was used (99). Proteins were diluted in water to approximately 1mg/ml and fluorescence emission at 420nm was measured after excitation at 350nm using either a LS5-B Perkin-Elmer Luminescence Spectrometer (fix scale=1, slit width=10nm) or an Aminco-Bowman (model J4-8960) Spectrophotofluorometer (emission slit=1mm, excitation slit=2mm). The latter fluorimeter was calibrated daily using a quinine sulphate solution prepared by dissolving quinine sulphate (1ug/ml) in 0.1M H₂SO₄. A plot of fluorescence against concentration of glycated protein (0-5mg/ml) was prepared to check limits of linearity (fig 2.8). The glycated BSA was prepared by incubating BSA (6 mg/ml) in 0.5M glucose or fructose for 16 and 11 days respectively at 37°C in 0.05M sodium phosphate buffer/3mM sodium azide, pH 7.4 (section 2.1). All measurements for samples were made in duplicate and the results expressed as either arbitrary units/mg protein or as relative fluorescence.

2.9.2: Crosslinked-AGE

These AGE were detected by their crosslinking on SDS gels after electrophoresis. Gels were stained with Coomassie Blue R and bands were quantified using laser densitometry.

The crosslinking was expressed as percent oligomer and calculated from;

$$\frac{\text{sum of intensities of polymer bands}}{\text{sum of intensities of polymer + monomer bands}} \times 100$$

Other gels were silver stained for a 100 fold greater sensitivity and used to

detect fragmentation products.

SDS Polyacrylamide Gel Electrophoresis: SDS gels (10%) were prepared according to the method of Laemmli (147). An LKB vertical slab gel electrophoresis cell was used to make gels of size 14 X 18cm and 1.5mm thick. The constituents required to make a 10% gel were;

Resolving gel: 10% acrylamide, 0.33% bisacrylamide, 0.375M Tris-HCl, pH 8.8, 0.1% SDS, 0.075% ammonium persulphate and 50ul TEMED per 100ml of resolving gel solution.

Stacking gel: 3.75% acrylamide, 0.1% bisacrylamide, 0.125M Tris-HCl, pH 6.8, 0.1% SDS, 0.075% ammonium persulphate and 75ul TEMED per 100ml of gel solution.

The resolving gel solution was applied between the plates till the level was approximately 2/3 the way up and then a small layer of water was poured on top. The gel was allowed to polymerize for 30 mins before pouring off the water. Stacking gel was layered on top and the combs inserted. The gel was allowed to set overnight at 4°C.

Sample preparation: Protein sample was treated with an equal volume of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue) and boiled at 100°C for 5 mins. Concentration of protein was adjusted so that there was approximately 2mg/ml after diluting with sample buffer. In experiments in chapter 9, the sample buffer was 0.05M potassium phosphate, pH 7.4, 5% dithiothreitol and 2% SDS.

Markers: Molecular weight markers ranging from 14 to 66KD (stock no SDS-6) were used for lysozyme and RNase gels whereas for albumin the markers ranged from 29 to 205KD (stock no SDS-6H). Both markers were from Sigma, Poole, Dorset, UK.

Electrophoresis: Samples of volume 10ul were loaded in the sample

wells followed by 50ul of the running buffer (0.025M Tris, 1.92M glycine, and 1% SDS, pH 8.3). The electrophoresis cell was placed in a LKB 2001 vertical electrophoresis unit attached to a cooler (LKB 2209 Multitemp). Using a LKB 2197 power supply, 150V was applied for 5 hours.

Fixing of gels: Coomassie Blue R staining, gels were fixed in 10% TCA overnight whereas for silver staining, gels were fixed in 50% ethanol and 10% acetic acid overnight.

Coomassie Blue R staining: Gels were gently stirred for 30 mins in Coomassie Blue R stain (0.25% Coomassie Blue R, 9.2% acetic acid and 45.4% methanol). Gels were destained in 7% acetic acid and 5% methanol with several changes.

Silver staining: After fixing the gels overnight, they were transferred to a dish containing 10% glutaraldehyde for 30 mins with gentle shaking. The gels were then rinsed in distilled water for 20 mins followed by rinsing in 15% ethanol with 3 changes over 10 mins. Gels were soaked for 10 mins in silver solution (0.077% NaOH, 0.014% NH_3OH and 0.78% AgNO_3). For 6 mins, gels were washed in distilled water and then placed in developing reagent (0.001% formaldehyde, 9.5% ethanol and 5% citric acid) for 10-15 mins till the bands became visible. To stop development, gels were rinsed in distilled water and stored in 15% ethanol.

Photography: Gels were photographed using a Ilford PanF ISO 50 B&W film (Ilford Ltd, Mobberley, Cheshire, UK) in a Cannon A1 camera with a Cannon FD lens 50mm f/1.4. The prints were developed on Ilfospeed 5 photographic paper to give a good contrast.

Scanning of Gels: Gels were scanned using a LKB Ultrosan XL laser densitometer and dried using an LKB 2003 slab gel dryer.

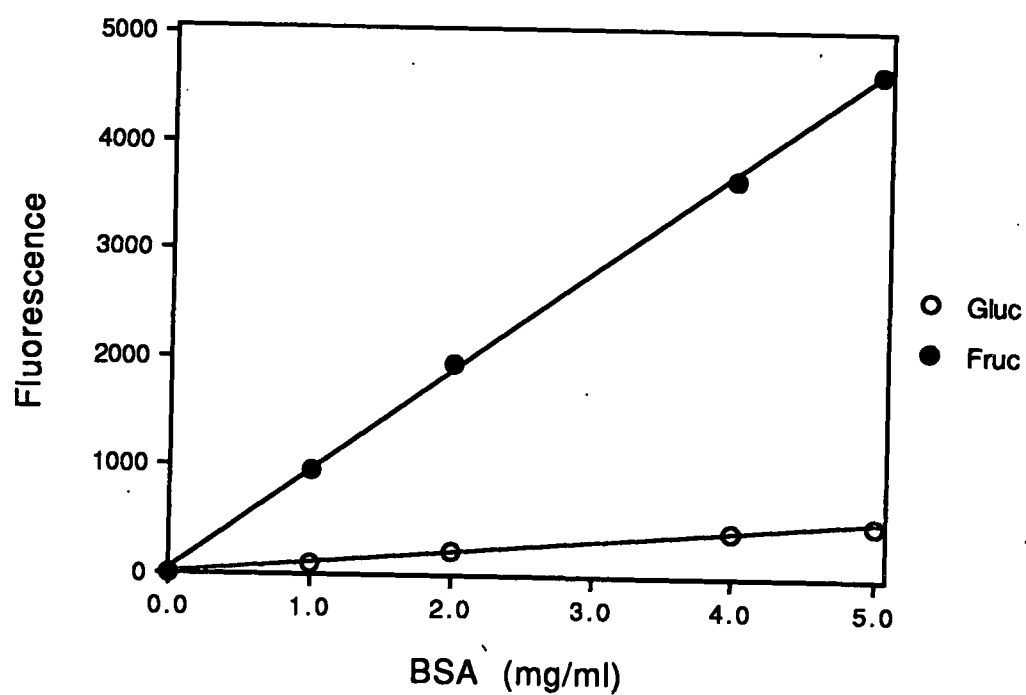


Fig 2.8: Effect of protein concentration on fluorescence of glycated BSA

2.10: Chemicals and Reagents

The chemicals and reagents used in this work were obtained from the following sources;

Proteins

Lysozyme (egg white)	Sigma Chemical Company,
Ribonuclease A	Poole,
Collagen (bovine achilles tendon type I)	Dorset (UK).
Human serum albumin (fatty acid free)	
Bovine serum albumin (fatty acid free)	
Bovine serum albumin (fraction V)	BDH Limited,
	Poole,
	Dorset (UK).

Enzymes

Collagenase	Sigma Chemical Company,
	Poole,
	Dorset (UK).
Proteinase K	Boehringer Mannheim,
	Lewes,
	East Sussex (UK).

Chemicals

N-acety-L-lysine-N-methylamide	Acetylsalicylic acid
Aminoguanidine	L-lysine
N-acetyl-L-cysteine	Sodium cyanoborohydride
Thiobarbituric acid	Galactose
Coomassie Blue R	Sorbitol
Trinitrobenzene sulphonic acid	Ketoglutaric acid

All of the above chemicals were obtained from;

Sigma Chemical Company,

Poole,

Dorset (UK).

All other reagents were of the highest purity available (Analar grade) and obtained from;

BDH Limited,

Poole,

Dorset (UK).

CHAPTER 3

Development of Glycation Microassays

3.1: Introduction

Measurement of glycated proteins such as Hb and albumin is of interest in clinical laboratories for the assessment of blood glucose control in diabetics. Glycation of long lived proteins such as collagen has been implicated in the secondary complications of diabetes and ageing and measurement of such proteins is of interest in research laboratories.

In this project model proteins such as albumin, lysozyme and ribonuclease were used and my requirements for a glycation assay were that it should; (i) be suitable for absolute values and reproducible, (ii) require only small amounts of protein, (iii) be suitable for large numbers of samples and (iv) be relatively simple and inexpensive. Of the glycation assays listed in section 1.8 of chapter 1, the furosine method is unsuitable since it requires a HPLC column and has a poor yield of only 30%. The borohydride method is laborious, non specific and unsuitable for large number of samples. The resins required for the phenylboronate method are expensive and electrophoretic methods are only suitable for Hb.

Of all the simple colorimetric assays for glycation, the periodate method has the advantage in that it is stoichiometric and suitable for absolute values. It quantifies the formaldehyde (HCHO) released by periodate oxidation of C-1 hydroxyls in the AP form of glycated protein.

Amadori product \longrightarrow HCHO \longrightarrow chromophore (DDL)

This HCHO is converted to the chromophore diacetyldihydrolutidine (DDL) and in the original method of Gallop et al (43) , this was detected fluorimetrically. The original Gallop procedure requires a fluorimeter, between 1-5mg of protein per assay tube, has a yield of DDL (determined in this study) of less than 70% and the calibration graph is linear only upto 40 nmols of DDL. The assay is time consuming especially when large numbers of samples are to be processed and this is probably one of the major reasons why the procedure is not in use in routine clinical laboratories. Theoretically there are a number of factors which could affect yield of DDL; (i) inadequate oxidation of AP to HCHO, (ii) loss of HCHO during removal of protein in the zinc sulphate precipitation step and (iii) inadequate conversion of HCHO to DDL. These factors were investigated and the procedure was modified to improve yield of DDL. The procedure was also scaled down and adapted for use with a microplate reader.

Another colorimetric assay, the TBA method has been used in some routine laboratories but has largely been discontinued since it is time consuming, cumbersome and unlike the periodate assay is non-stoichiometric and therefore only suitable for comparative purposes and not absolute values. However, unlike the periodate assay, the TBA method can be used to measure glycated collagen since collagen crosslinks interfere in the former assay. Although the TBA method has been superseded by other methods for assaying glycated Hb and albumin, it is still a simple, convenient and inexpensive method for measuring glycated collagen. It has advantages over the furosine method for assaying glycated collagen in that it is simpler, does not require expensive equipment or reagents and is faster and more appropriate for processing large numbers of samples. Furthermore, the protein does not have to be hydrolysed to its constituent amino acids. The TBA assay requires some 10mg of protein

and the yield of HMF varies between 10% (42) to 80% under optimum conditions (148) and depends critically on the protein concentration (149). In the initial stages of this project I was intending to work on collagen (which unfortunately had to be abandoned later) and required a simple rapid assay for measuring glycation. The autoclave version of the TBA method of Parker et al was chosen since it has the advantage in that it only requires one hour of boiling compared to earlier versions which involved between 4-6 hours and therefore were very time consuming (144). This Parker method was further modified to increase sensitivity and yield of HMF and the method was adapted for use with a microplate reader so that it was faster and less cumbersome. All these objectives were achieved to give a simple, rapid microassay suitable for measuring glycation of collagen as well as other proteins. Free sugar will interfere in the TBA method and have to be removed prior to assay. Glycoproteins with acid labile glycosidic bonds have been reported to interfere in this assay (42) and present a major problem in interpreting results.

3.2: Aims

- 1: To determine and improve yields of DDL in the periodate assay of Gallop et al by investigating the different steps at which losses may occur using its published molar absorbance.
- 2: To improve the speed and sensitivity of the periodate assay by minimising quantities and adapting for use with a microplate reader.
- 3: To improve the speed and sensitivity of the TBA method of Parker et al by minimising quantities and adapting for use with a microplate reader.
- 4: To investigate the relationship between protein concentration and absorbance of chromophore using a defined glycated protein for both TBA and periodate microassays.

- 5: To determine the correlation coefficient between the periodate and TBA microassays and compare the apparent extent of glycation of HSA by both assays.

3.3: Experimental Protocol

In Vitro Glycation: Fast glycation involved incubating collagen (40mg/ml) and BSA Fatty acid free (10mg/ml) in 0.5M glucose for upto 20 days at 37°C in 0.05M sodium phosphate buffer/3mM sodium azide, pH 7.4 (section 2.1). Unbound sugar and labile SB were removed by extensive dialysis against water (section 2.2). Collagen was digested (section 2.3) and both proteins stored frozen.

Protein Concentration: This was determined using the Pierce BCA protein assay reagent (section 2.4.2).

Yield of DDL in Periodate Method: Fructose standards prepared in distilled water were treated exactly as in the original method of Gallop et al (section 2.8.1) or as in the new microassay (section 2.8.2). Formaldehyde standards were also prepared and incubated directly with the detection reagent (FDR). The yield of DDL for standards treated according to the Gallop procedure was determined by measuring the absorbance at 412 nm on a PYE Unicam Spectrophotometer using molar absorbance of DDL as $8,000 \text{ l mol}^{-1}\text{cm}^{-1}$ (44). The nearest wavelength of the filter for microplate reader was 405nm, and using the spectrophotometer to compare the absorbance at 412nm and 405nm, the molar absorbance at 405nm was calculated as $7780 \text{ l mol}^{-1}\text{cm}^{-1}$. This value was used to determine yield of DDL in the microassay.

Yield of HMF during Hydrolysis in the TBA Microassay: Two sets of HMF standards were prepared and treated exactly the same (section 2.8.3) except that in one set the heating step was omitted. Assuming complete

conversion of HMF to chromophore, the difference in the absorbance values for the chromophore between the two sets was used to calculate the percentage of HMF lost during heating.

3.4: Results

A linear relationship was obtained when the absorbance at 412nm for DDL was plotted against number of moles of fructose as shown in fig 3.1 The yield of DDL from fructose was calculated as 67.5% using its molar absorbance as 8,000 l mol⁻¹cm⁻¹ (table 3.1).

A linear relationship was also obtained when chromophore was produced directly from HCHO in the same method (fig 3.1) and the yield of DDL from HCHO was 100% (table 3.2) suggesting that this second step of the assay goes to completion.

For the periodate microassay, there is a linear relationship between the production of DDL and fructose or HCHO standards (fig 3.2) and the yield from fructose is now over 90%.

In the TBA microassay, the loss of HMF during 1 hour hydrolysis was no greater than 13% ie;

HMF standards (nmols)	Percentage loss of HMF
10	11
30	13
50	12

The coefficient of variation (CV) of a method refers to the size of the random errors and the reproducibility of the measurements (ie the ability to provide the same value on multiple analysis). As the CV improves, its value approaches

zero. Using HSA, the extent of glycation was determined by both methods (table 3.3) and the CV calculated.

In the range 0-0.3mg of protein per well, absorbance was linear with concentration of glucated BSA for the periodate microassay (fig 3.3). Using glucated collagen (0-2mg), a linear relationship was also obtained for protein concentration against absorbance in the TBA microassay (fig 3.4).

A correlation coefficient (r) of 0.94 was obtained for 22 BSA samples glucated to different extents in vitro when measured by the periodate and TBA microassay (fig 3.5).

Fructose standards (nmols)	Observed DDL (nmols)	Percentage yield of DDL
10	6.75	67.50
20	13.50	67.50
30	20.25	67.50
40	27.00	67.50
50	33.75	67.50

Table 3.1: Percentage yield of DDL from fructose standards (0-50nmols) in the periodate assay (Gallop procedure)

HCHO standards (nmols)	Observed DDL (nmols)	Percentage yield of DDL
10	10.13	100
20	20.25	100
30	30.38	100
40	40.50	100
50	50.60	100

Table 3.2: Percentage yield of DDL from HCHO standards (0-50nmols) in the periodate assay (Gallop procedure)

Sample	P.I (moles/mole)	TBA (moles/mole)
1	0.45	0.35
2	0.44	0.36
3	0.44	0.37
4	0.44	0.35
5	0.45	0.35
Mean	0.44	0.35
S.D	0.005	0.008
C.V	1.1%	2.25

Table 3.3: In vivo glycation of HSA (expressed as moles AP/mole protein) as measured by the periodate and TBA microassays. The results are given as mean +/- SD (n=5).

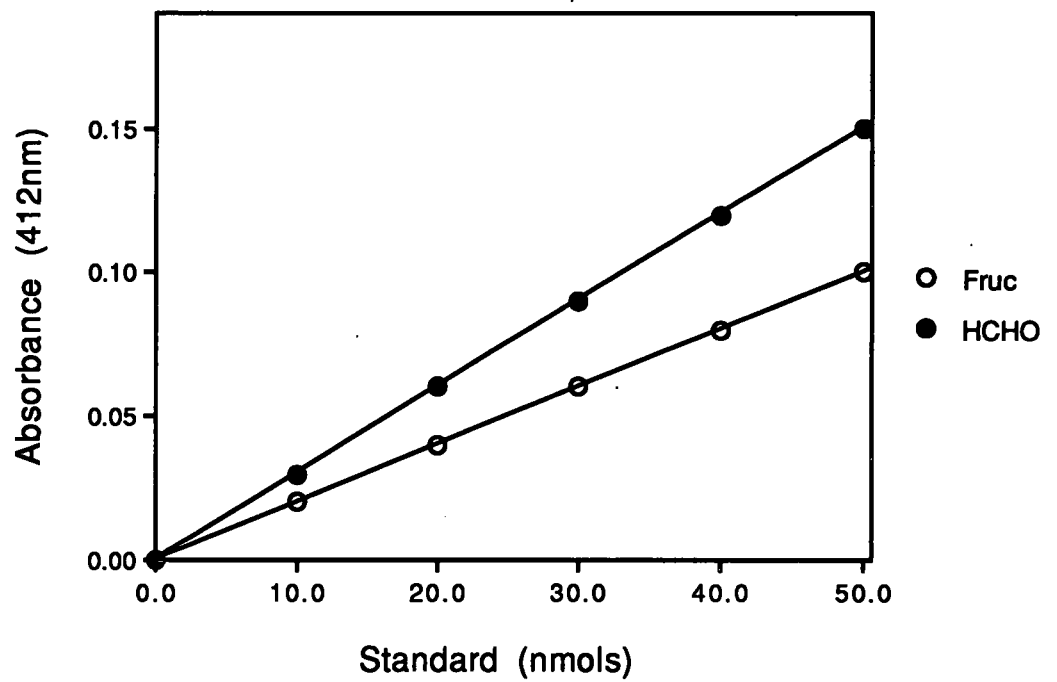


Fig 3.1: Production of DDL from fructose and formaldehyde standards in the periodate assay (Gallop procedure)

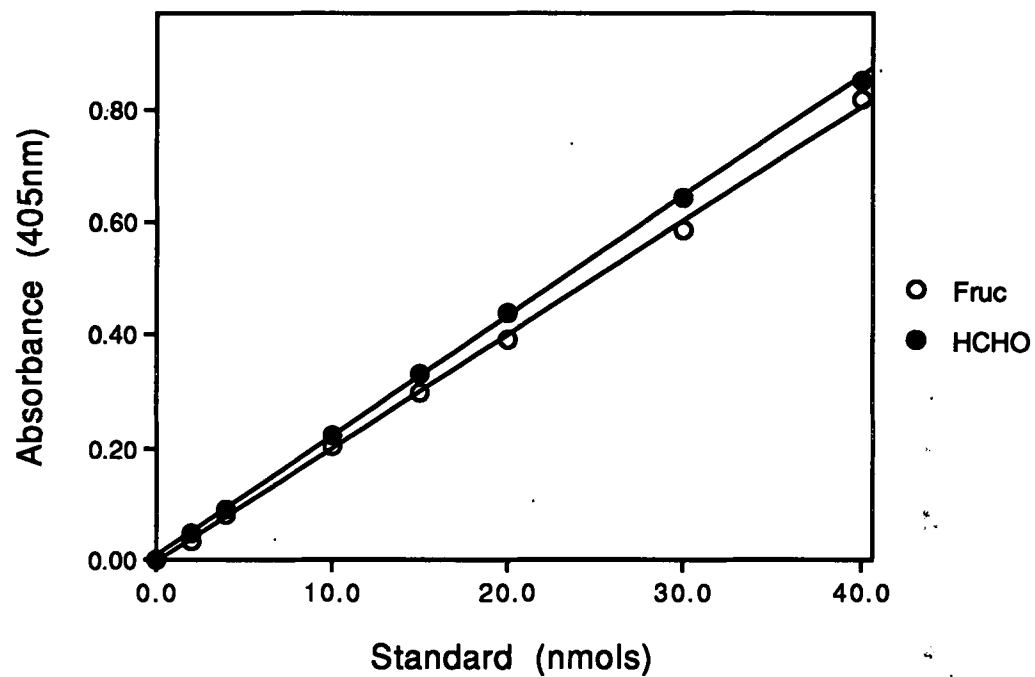


Fig 3.2: Production of DDL from fructose and formaldehyde standards in the periodate microassay

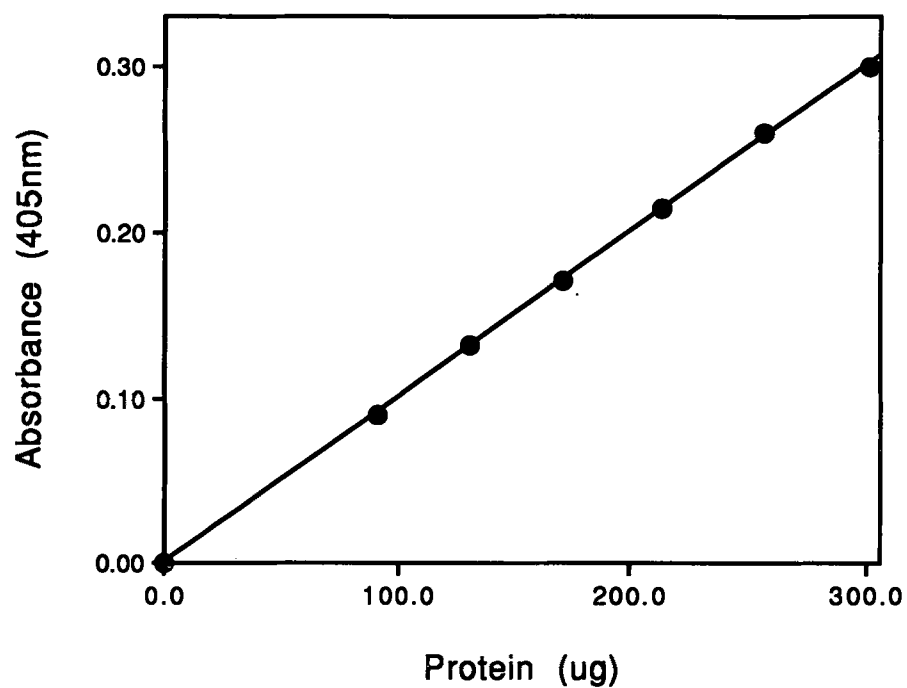


Fig 3.3: Effect of increasing protein (glucated BSA) on production of DDL

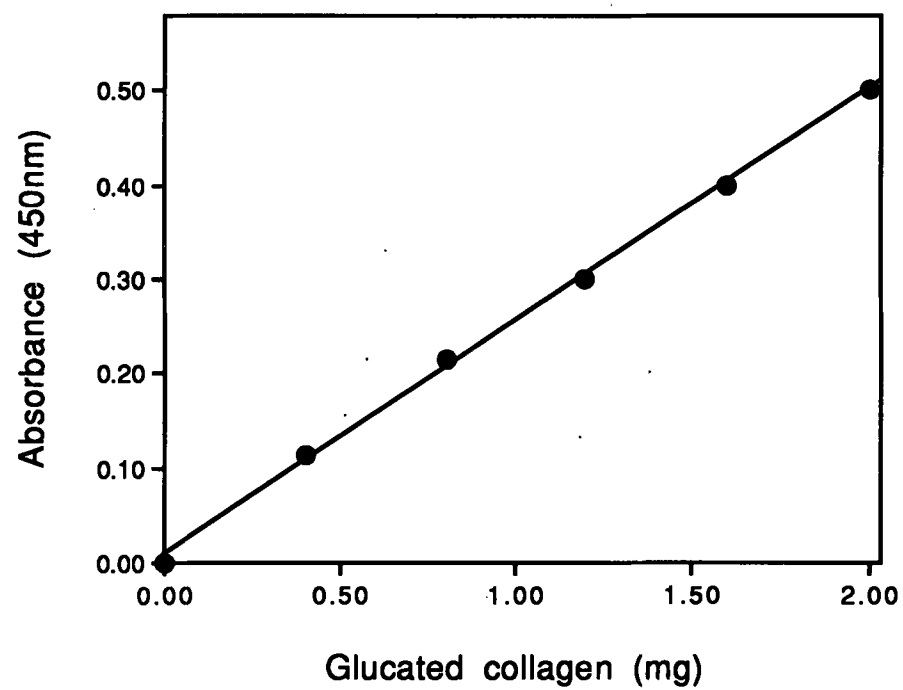


Fig 3.4: Effect of increasing protein (glucated collagen) on production of chromophore in the TBA microassay

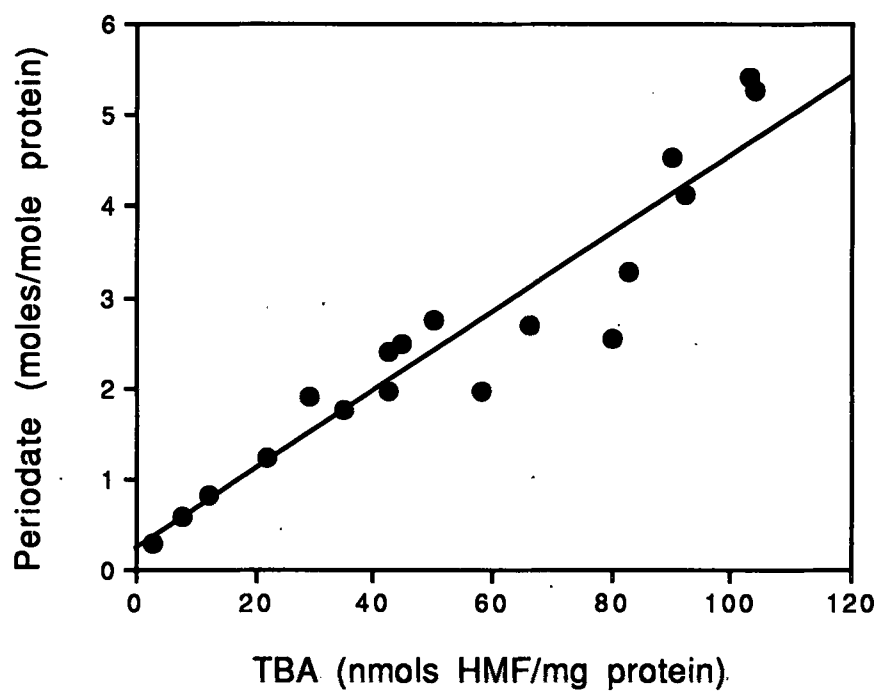


Fig 3.5: Comparison of glucated BSA measured by the periodate and TBA microassay

3.5: Discussion

In this study, I report use of the periodate method in conjunction with a spectrophotometer whereas the original Gallop procedure relied on use of a fluorimeter. Fructose resembles the AP form of glucated protein in having a C-2 carbonyl group in the straight chain configuration, but existing predominantly in the ring form. Like the AP, it liberates 1 mol of HCHO per mole on periodate oxidation, and can therefore be used as a standard for the AP. In the Gallop procedure, the calibration graph is linear upto 40nmols of fructose and then starts to deviate from linearity, presumably due to quenching (fig 2.4, chapter 2). No such deviation is seen when DDL is measured by its absorbance, and the graph is linear upto 50nmols (fig 3.1) and above (not shown). Yield of DDL from fructose standards was 67% (table 3.1), and theoretically this yield may be reduced at any of 3 stages;

- 1: by incomplete oxidation and cleavage of C-1 to produce HCHO
- 2: by adsorption or trapping of free HCHO in the zinc periodate precipitate
- 3: by incomplete conversion of HCHO to DDL

The yields of DDL in this last step were measured by incubating standard HCHO solutions with FDR (table 3.2). A 100% yield of DDL from HCHO was obtained and this suggests that loss of HCHO occurs during oxidation and/or centrifugation. The procedure of Gallop et al was scaled down keeping everything in the same proportion so that it could be used with a microplate reader. Fig 3.2 shows that with the microassay procedure, absorbance is linear for 5-40nmols of fructose per well. As little as 2nmol can be readily detected (giving absorbance readings of 0.04). Concentrations below this are not detected probably due to the short path length calculated as 0.88cm per 300ul of solution and also due to the yield of HCHO from fructose. Loss of HCHO in

the zinc periodate precipitate has been reduced by increasing speed of centrifugation from 1000g in the Gallop procedure to 9000g in my microassay. A greater proportion of HCHO containing solution can thus be removed as supernatant liquid and converted to DDL. This modification allowed the overall yield (from fructose to DDL) from 67% with the Gallop procedure to 90% with the microassay. This compares favourably with yields in other methods eg 30% for the furosine assay (42) and 80% for the TBA assay under optimum conditions (148). The sensitivity of the assay has been improved by ten fold ie requires only 0.1mg of protein compared to at least 1mg required by the Gallop procedure. Furthermore a fluorimeter is no longer required and since the DDL is being measured by its absorbance, the calibration graph is linear upto 100nmols of fructose. Free sugars and SB interfere in the periodate assay but can be eliminated by extensive dialysis prior to assay. Glycoproteins will not interfere in this assay unless they carry chain terminal reducing sugars or sialic acid derivatives.

In the range of 0-0.3mg of protein per well, absorbance was linear with concentration of glucated BSA (fig 3.3) and the CV of 1.1% compares favourably with the CV of 2.2% obtained with the Gallop procedure (43).

The TBA method of Parker et al has been modified and adapted for use with a microplate reader. The nett amount of HMF available for chromophore formation depends on the balance between its production and loss during the heating process. Parker et al reported 30% destruction of HMF after 1 hour of heating in an autoclave whereas in the microassay no more than 15% loss of HMF occurred. Furthermore, HMF yield also varies with the amount of protein used and is greater with less protein (149). Since the microassay only requires 1mg of protein compared to 10 mg in the Parker method a better yield of HMF would

be expected. Using in vitro glucated collagen, it was found that in the range 0-2mg, absorbance was linear with concentration of protein. A CV of 2.25% is obtained with the TBA microassay and this compares with the CV of 1.65% obtained with the Parker method (144).

Extent of glycation of HSA measured by both TBA and periodate microassays were compared to that in the literature ie;

Study	Method	Moles AP/mole
Schleicher et al (90)	Furosine	0.24
Baynes et al (150)	Borohydride	0.29
Olufemi et al (151)	Periodate	0.37
Walton et al (152)	Periodate	0.49
This study	Periodate	0.44
This study	TBA	0.36

HSA was chosen in this study since it is easy to compare values obtained by these assays with those in the literature. The value of 0.44 moles/mole of protein compares well with that obtained by the Walton group using the original method of Gallop et al. The value obtained by the TBA microassay also compares favourably with the other methods. A correlation coefficient (r) of 0.94 was obtained for the two methods which suggests that the periodate microassay compares well with the more established TBA method. Both microassays have the advantage that they are faster to perform ie with the microplate reader upto 94 samples can be read in 40 seconds in the last step and less reagent is required therefore saving costs. The periodate microassay was chosen for my work since it is stoichiometric, more reproducible than the TBA method and takes less time to perform.

CHAPTER 4

Glycation and AGE Formation in Proteins by Different Sugars

4.1: Introduction

Almost all the other reducing sugars are more reactive than glucose but have not been thoroughly investigated because of their minute concentrations in vivo. The study of these sugars is important since their rarity could be compensated by their greater reactivity. Under certain circumstances, levels of these sugars may be high eg high levels of galactose during galactosaemia. Comparative studies so far have looked at differences in the rate of SB formation and to my knowledge a comprehensive study on the differences in rates of AP and AGE production have not yet been undertaken. Such comparative studies may provide an insight into how structural differences between sugars may affect rates of the Maillard reaction and could raise new questions regarding the role of these other sugars in ageing and diabetic complications.

Using radiolabelled sugars, Bunn et al have shown that the initial reaction (ie SB formation) between a protein and sugar depends on the percentage of sugar in the acyclic form and on the reactivity of its carbonyl group (11). Also the reaction rate is inversely proportional to the number of C atoms in the sugar, being lowest for hexoses and highest for trioses like glyceraldehyde. Taking the above considerations into account then the order of SB formation by the four sugars used in this study would be expected to be ribose then fructose, galactose and glucose. Indeed this is the case and has already been demonstrated by Bunn et al (11). In this chapter rates of AP and AGE formation after incubating BSA in different sugars were compared to determine whether

the order of AP and AGE formation by different sugars was similar to their order of SB formation. The rate of AP formation after glycation of BSA in vitro by different sugars was measured using the periodate microassay. Sugars which have the highest comparative rates for AP formation might be expected to have similar comparative rates for fluorophore formation. However this assumes that post-Amadori reactions are the same for proteins glycated by different sugars and this is very unlikely.

The reactivity of free amino groups within a protein can also vary depending on their accessibility within the protein structure and on their pK. Furthermore certain amino groups are more susceptible to glycation because they are adjacent to acidic groups (12) which can catalyse the Amadori rearrangement. Since it is known that the susceptibility of different proteins to glycation may vary, the effect of different sugars on fluorescence formation in other proteins ie lysozyme and RNase was also investigated. The susceptibility of a protein to form different types of AGE ie fluorescent or crosslinked could vary ie some proteins may form crosslinks more readily than others and vice versa. To investigate whether this was true, equimolar concentrations of lysozyme and RNase were reacted under identical conditions and then compared for crosslinking and fluorescence formation.

High non-physiological concentrations of sugars have been used in this study because glycation and the Maillard reaction are slow processes and use of lower concentrations would have demanded increased incubation periods with a higher probability of bacterial contamination. BSA, lysozyme and RNase were chosen as model proteins because they are soluble, relatively inexpensive and already well characterised with respect to glycation.

4.2: Aims

- 1: To determine whether the comparative rate of AP formation by different sugars was similar to their known rates of SB formation, using BSA as a model protein in vitro.
- 2: To determine whether rate of fluorescence generation by different sugars in vitro was similar to their known rates of SB formation, using BSA as a model protein.
- 3: To determine whether crosslinked and non-crosslinked-AGE form independently of each other, crosslinking and fluorophore formation in lysozyme and RNase was compared.
- 4: To investigate whether the effect of different sugars on fluorescence generation is similar to their effect on crosslinking using lysozyme and RNase as model proteins.

4.3: Experimental Protocol

In Vitro Glycation: BSA fraction V (10, 20 and 40mg/ml), lysozyme (10mg/ml) and RNase (5mg/ml) were incubated at 37°C in 0.5M sugar for upto 16 days in 0.05M sodium phosphate buffer/3mM sodium azide (section 2.1). Equimolar concentrations (2mM) of lysozyme and RNase were also incubated in 50mM ribose for upto 10 days under the above conditions. Proteins were dialysed against distilled water (section 2.2) and stored frozen.

Protein Concentration: This was determined using the Pierce BCA protein assay reagent (section 2.4.2).

Measurement of Glycation: AP levels in glycated BSA were measured using the periodate microassay (section 2.8.2).

Measurement of AGE: AGE were measured by their crosslinking on SDS gels (section 2.9.2) or fluorescence (section 2.9.1).

4.4: Results

The periodate method used to measure extent of glycation of BSA induced by the different sugars quantitates only the AP since the labile SB has been removed by extensive dialysis prior to assay. Extent of ribation of BSA after 15 days of incubation was almost 4 times greater than glucation (fig 4.1). The order in which the sugars form AP is ribose > glucose > galactose > fructose (fig 4.1) and the increase in AP levels as a function of time is biphasic.

Solutions of BSA incubated in sugars developed a yellow colour which gradually darkened and became brown with time. In prolonged incubations over several months a dark brown precipitate was detectable at the bottom of tubes (results not shown). The order in which the different sugars caused these visible changes was ribose > fructose > galactose > glucose.

BSA incubated in all the different sugars generates fluorescence with the characteristic emission spectra described previously by Suarez et al (99). In the absence of sugar no rise in fluorescence is detectable suggesting that fluorescence is sugar-induced (see chapter 7 later). Rate of fluorescence formation is highest with ribose but unlike the rates of AP formation, the order of reaction is ribose > fructose > galactose > glucose (fig 4.2). In the ribose and fructose incubations nearly all the fluorescence is generated during the first 7 and 10 days respectively after which the fluorescence yields appear to reach saturation. In contrast, a more gradual increase in fluorescence occurs with glucated and galactated BSA which seem to be heading for the same saturation values.

In fig 4.3, in vitro glycation of BSA reduces its electrophoretic mobility and a broadening of the protein staining band occurs particularly with fructose. Under

these conditions, little or no polymerisation is seen. The BSA used was fraction V and contaminants ie mainly globulins are also visible on gel.

The order of fluorescence generation by the four sugars with lysozyme (fig 4.4) and RNase (fig 4.5) is similar to that for BSA ie ribose > fructose > galactose > glucose. Again fluorescence formation for both proteins incubated in ribose or fructose reaches saturation after about 7 days. For both lysozyme and RNase incubated in glucose and galactose, there appears to be a lag phase for 7 days before a rise in fluorescence occurs. Fig 4.6 shows the fluorescence generated by equimolar concentrations of lysozyme and RNase incubated in ribose. Fluorophores generated by ribated RNase form twice as fast as those by ribated lysozyme. However, when crosslinking of ribated lysozyme on SDS gels (fig 4.7) and ribated RNase (fig 4.8) was quantified, it was found that lysozyme crosslinked faster than RNase (fig 4.9). The polymerisation of lysozyme was 25% higher than that of RNase after 10 days of ribation whereas the fluorescence is only 46% of that generated by RNase. For lysozyme, a small percentage of protein (2-3%) was crosslinked in the controls even at zero time (fig 4.7) despite boiling of samples in mercaptoethanol prior to electrophoresis. Initially this crosslinked lysozyme was believed to be due to incomplete reduction of disulphide bonds. However, even treatment of samples using twice the concentration of mercaptoethanol (taken fresh from stock just before boiling) and increasing the period of boiling to 10 mins did not reduce this crosslinking. Similar crosslinking of lysozyme can also be detected in gels quantified by Shin et al (18). This value was therefore subtracted from the rest to correct for sugar-derived crosslinking.

The order in which different sugars induced crosslinking was studied using lysozyme and RNase since these proteins crosslink readily. For RNase, the

order in which the sugars induced crosslinking was ribose > fructose > galactose > glucose ie similar to that for the fluorescence. Fig 4.10 shows the gel for RNase glyated by different sugars for 12 days whereas fig 4.11 is the similar gel for lysozyme. Extensive glycation of RNase or lysozyme results in protein precipitation due to the formation of mellanoidins. Initially these were removed by centrifugation and the supernatant used for SDS-PAGE. Obviously this does not give a realistic representation of the true extent of crosslinking. For this reason, proteins were incubated for shorter periods of time so as to prevent formation of mellanoidins. No precipitation of protein glyated by 0.5M sugar for upto 12 days was detectable under the conditions used. RNase does not polymerise readily like lysozyme to give discrete oligomers ie there is a lot of protein between the bands for oligomers especially for ribated RNase (see fig 4.10). This is probably due to impaired SDS binding causing reduced electrophoretic mobility which is particularly high for ribated proteins since they are more extensively glyated. The portion of streak in lane F (fig 4.10) labelled monomer is of protein less than the molecular weight of dimer. These gels were quantified and the results are given in table 4.1. The figure of 45% polymerisation for ribated RNase is therefore only an approximation.

For lysozyme the order in which the sugars induced the highest crosslinking was fructose > ribose > galactose > glucose ie fructose appears to be a more powerful crosslinking reagent. This compares with the fluorescence results where ribose is clearly the most potent inducer of fluorescence.

The experiments were repeated twice (n=2) and similar results found each time. The most representative results are shown. Since the conditions for the experiments were not always identical, it has not been possible to analyse the results statistically.

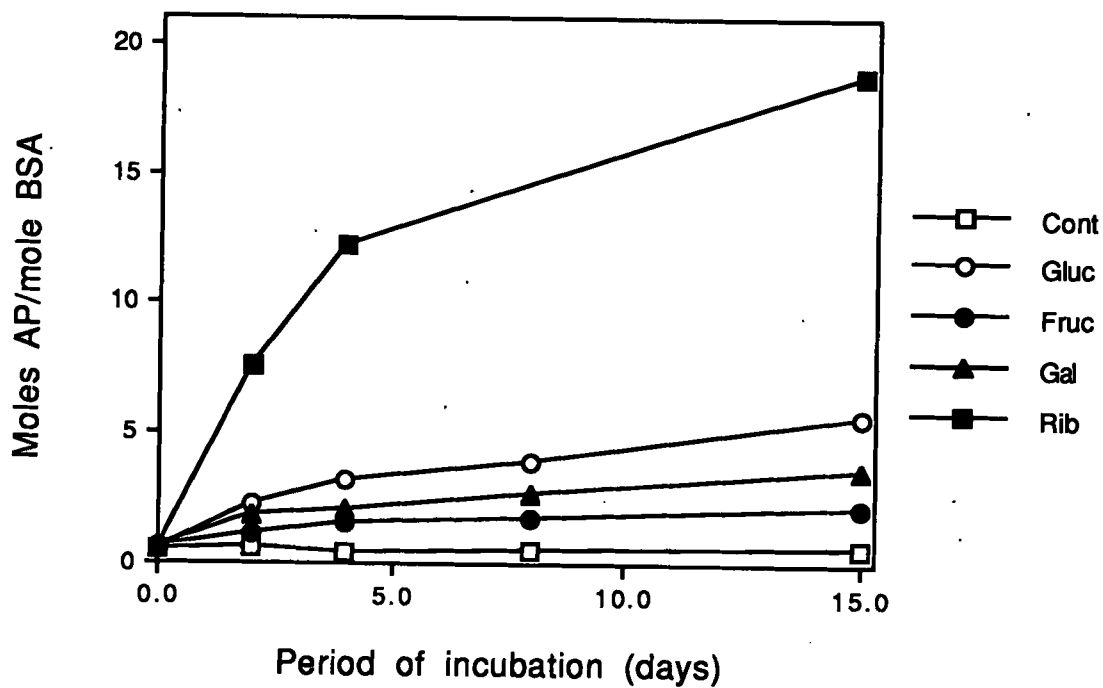


Fig 4.1: Apparent rates of AP formation after in vitro glycation of BSA (20mg/ml) by 0.5M sugar

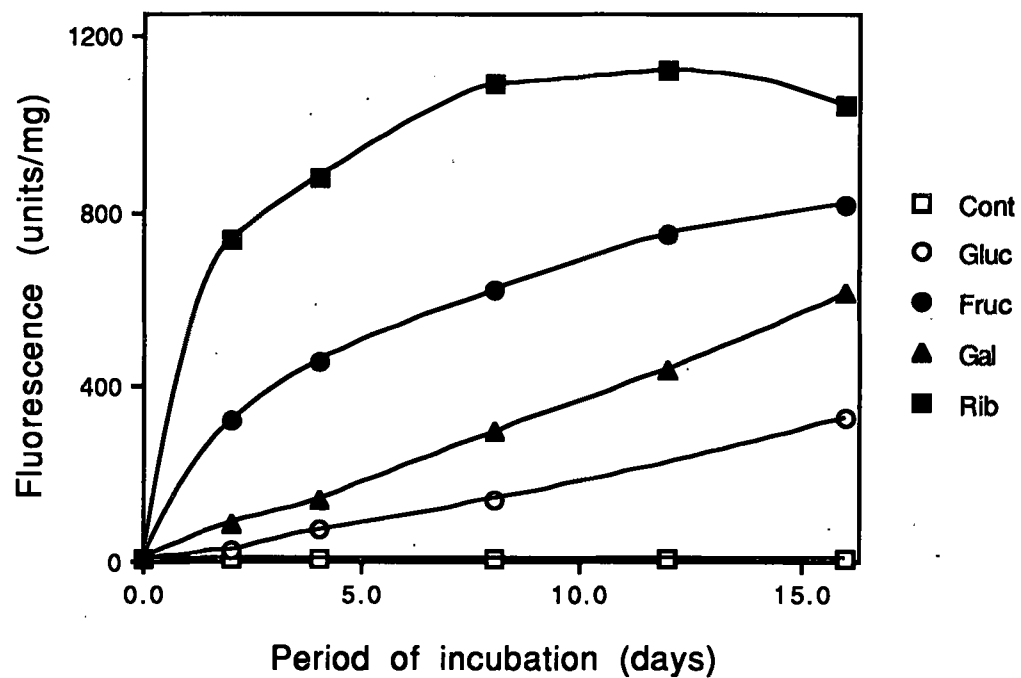


Fig 4.2: Fluorescence of BSA (10mg/ml) incubated in 0.5M sugar

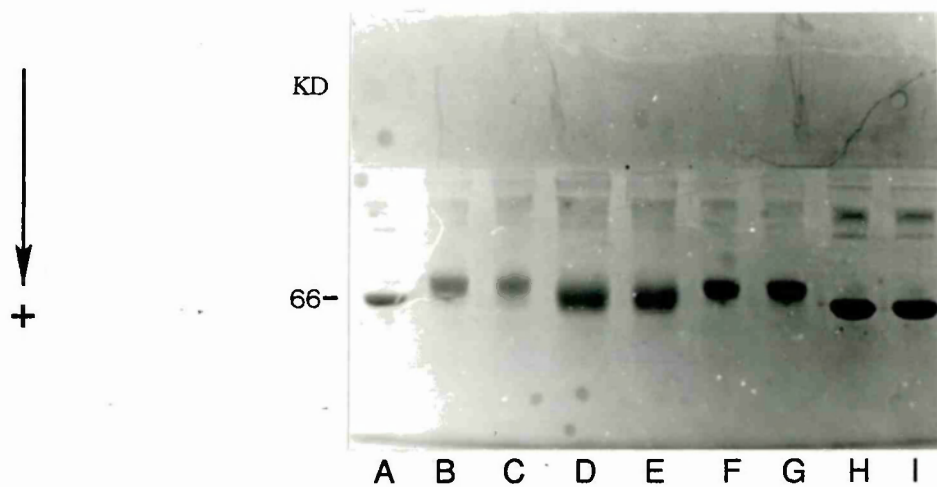


Fig 4.3: SDS-PAGE of BSA (40mg/ml) glycated in vitro for 16 days by 0.5M galactose (lanes B-C), fructose (lanes D-E) or glucose (lanes F-G). Non glycated BSA (lanes H-I) and marker for BSA (lane A) is also shown.

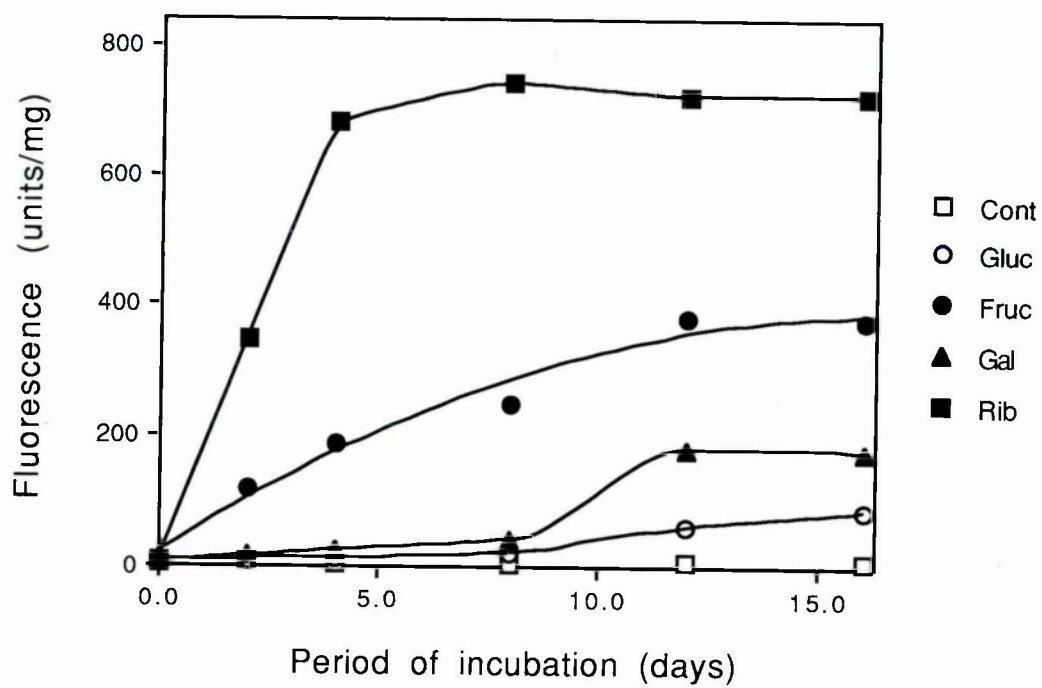


Fig 4.4: Fluorescence of lysozyme (10mg/ml) incubated in 0.5M sugar

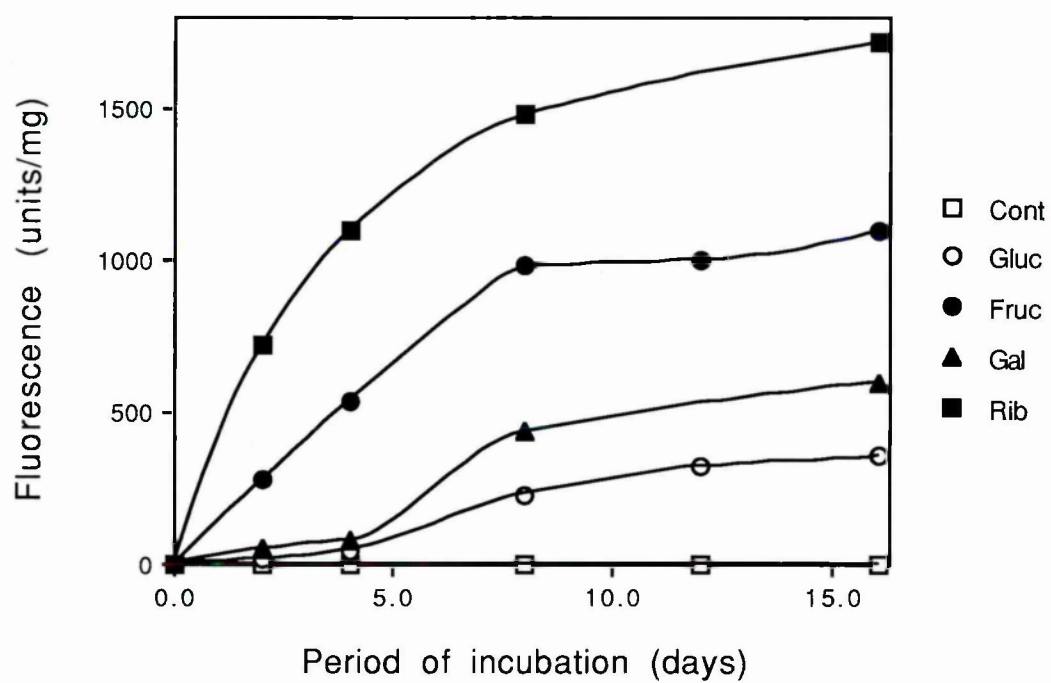


Fig 4.5: Fluorescence of RNase (5mg/ml) incubated in 0.5M sugar

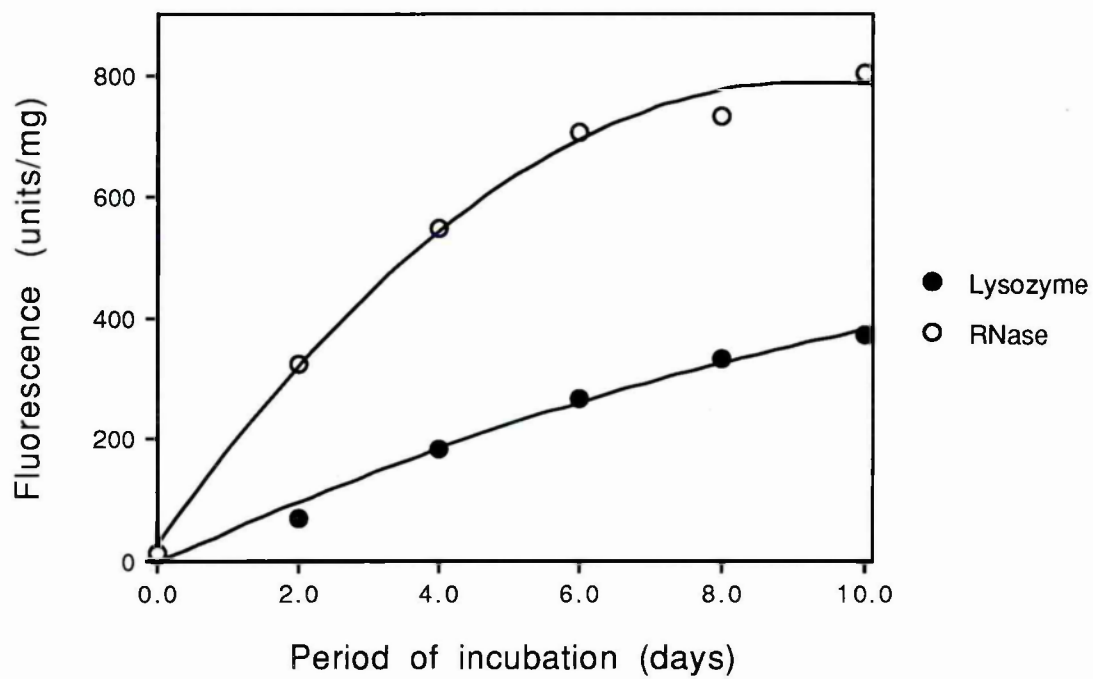


Fig 4.6: Fluorescence of lysozyme and RNase (2mM) incubated in 50mM ribose

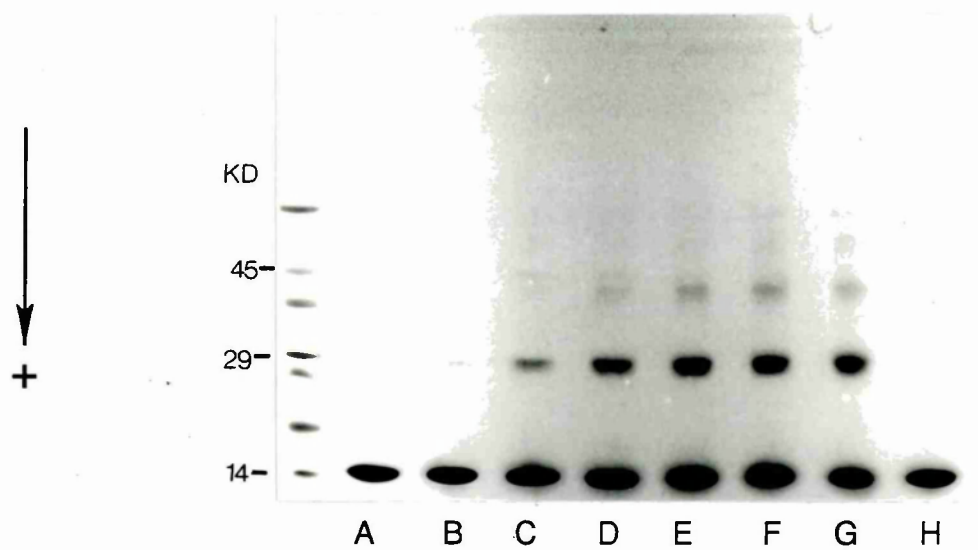


Fig 4.7: SDS-PAGE of lysozyme (2mM) glycated in vitro by 50mM ribose for 0, 2, 4, 6, 8 and 10 days (lanes B, C, D, E, F and G respectively). Also shown is lysozyme incubated in the absence of ribose for 0 (lane A) and 10 days (lane H).

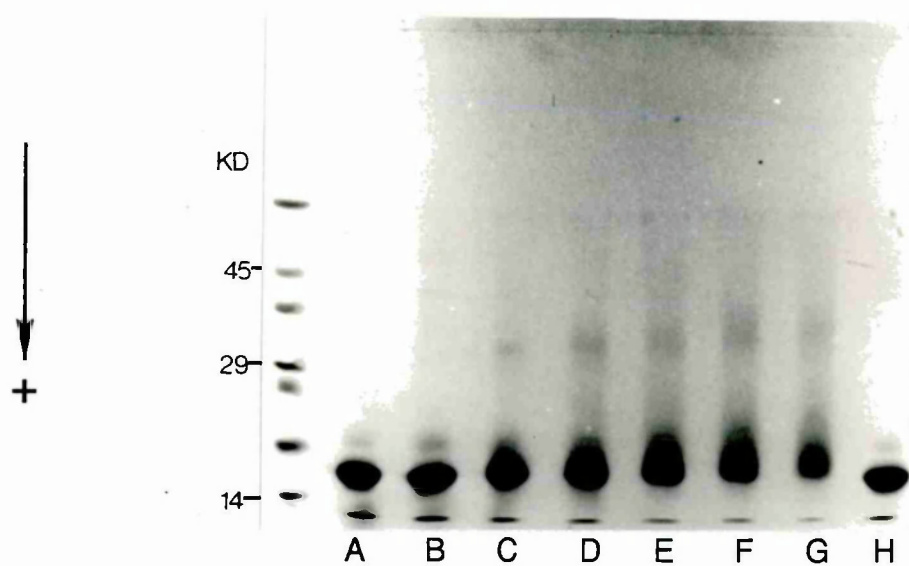


Fig 4.8: SDS-PAGE of RNase (2mM) glycated by 50mM ribose for 0, 2, 4, 6, 8, and 10 days (lanes B, C, D, E, F and G respectively). Also shown is RNase incubated in the absence of ribose for 0 (lane A) and 10 days (lane H).

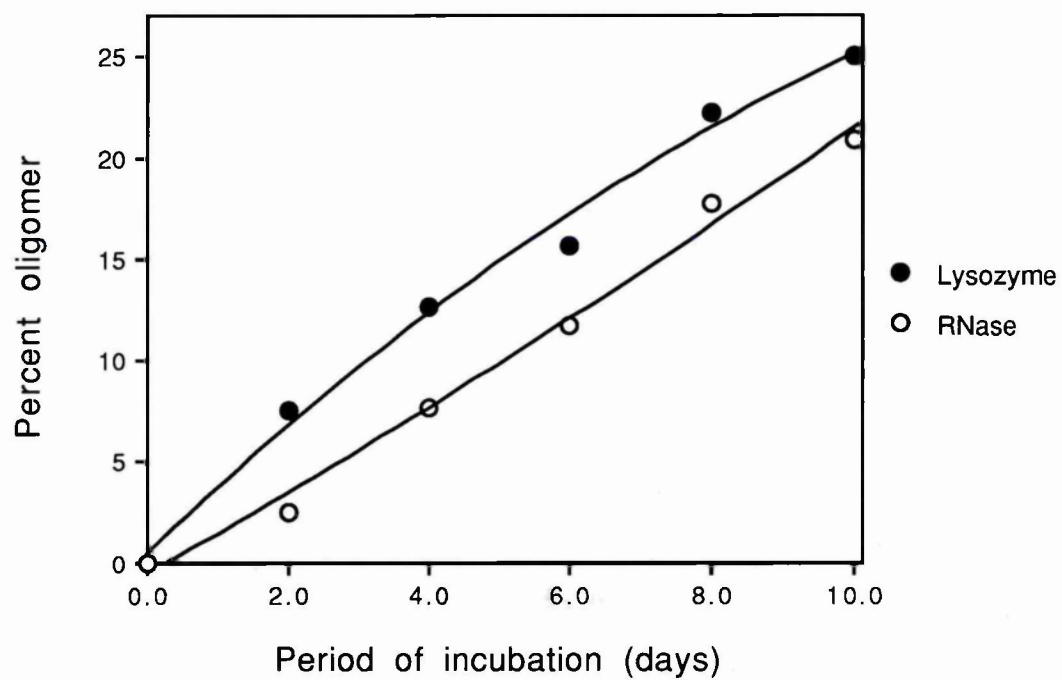


Fig 4.9: Crosslinking of lysozyme and RNase (2mM) incubated in 50mM ribose

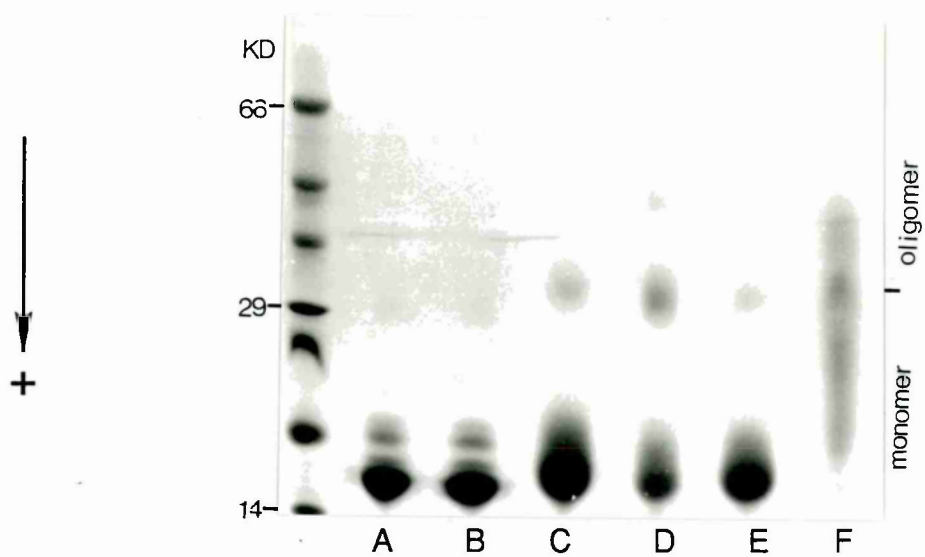


Fig 4.10: SDS-PAGE of RNase (5mg/ml) glycated in vitro for 12 days by 0.5M glucose (lane C), fructose (lane D), galactose (lane E) and ribose (lane F). Also shown is RNase incubated in the absence of sugar for 0 (lane A) and 12 days (lane B).

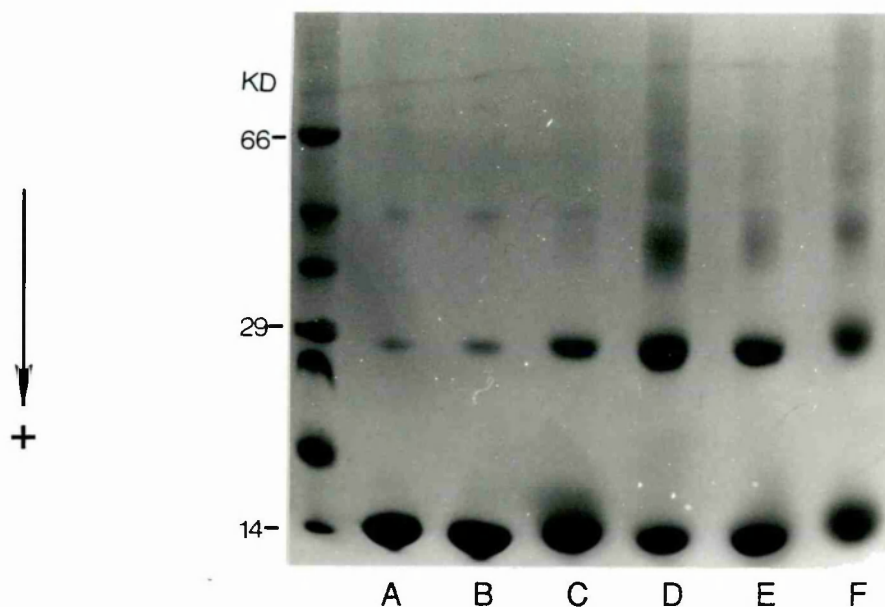


Fig 4.11: SDS-PAGE of lysozyme (10mg/ml) glycated in vitro for 12 days by 0.5M glucose (lane C), fructose (lane D), galactose (lane E) and ribose (lane F). Also shown is lysozyme incubated in the absence of sugars for 0 (lane A) and 12 days (lane B).

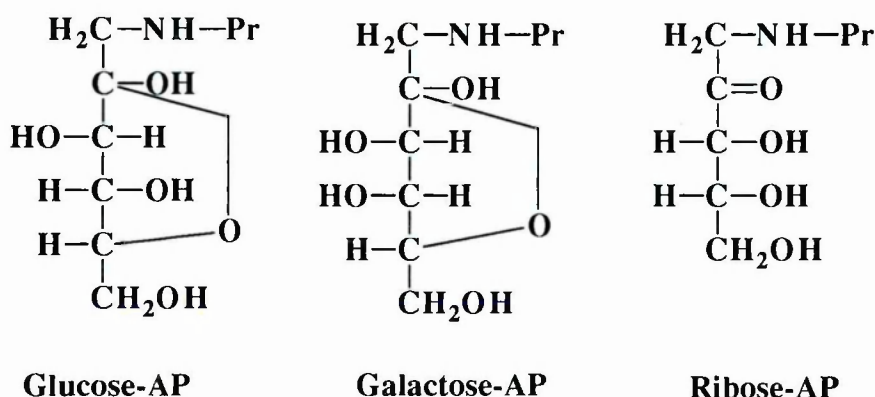
Protein	Control	Glucose	Fructose	Galactose	Ribose
RNase	0	4.9	21.4	5.8	45.2
Lysozyme	0	15.3	41.3	31.4	38.6

Table 4.1: Polymerisation of RNase and lysozyme glycated for 12 days by 0.5M
sugar

4.5: Discussion

If the Amadori rearrangement was identical for all sugars then their comparative rates of AP formation should be identical to their rates of SB formation. However, this does not appear to be the case in this study where the order of AP formation by different sugars is ribose > glucose > galactose > fructose (fig 4.1). There are two possible reasons for this. First post-Amadori reaction rates vary for different sugars for eg fructose has been reported to induce AGE formation upto 10 times faster than glucose probably due to greater reactivity of its aldehydic AP (99). As a result the steady state levels of fructose-derived AP may be low as they are rapidly converted to AGE. Greater reactivity of galactose-derived AP may also account for their low levels. Although galactose-derived AP would not be expected to be aldehydic in nature, a higher proportion may exist in the acyclic form compared to glucose-derived AP and therefore participate more rapidly in AGE formation. A second reason could be that AP derived from sugars other than glucose may not be detected by the periodate assay. Indeed, the fructose-derived aldehydic AP would not be expected to release HCHO upon periodate oxidation (99) and so in this study I am detecting less than half of the fructose-derived AP (see chapter 5 later). In contrast, galactose-derived AP would be expected to release HCHO upon periodate oxidation. Hitz et al have demonstrated using a radiolabelled technique that incorporation of galactose occurs at a faster rate than glucose even after excluding SB for several proteins including albumin (153). This group therefore suggested that galactose forms AP faster than glucose which would appear to conflict with my result. However, it is worth pointing out that sugars incorporated into AGE would also be measured with the radiolabelled technique but probably not detected by the periodate assay which accounts for the discrepancy between my results and those of Hitz et al.

In the case of ribose it appears that all the AP are being detected, as would be expected theoretically. Another study using phenylboronate affinity chromatography has also shown that ribose-derived AP form considerably faster than those from glucose (154). The expected chemical structures for glucose, galactose and ribose derived-AP are given below;



By applying the Malaprade's rule (see section 1.8.2 and fig 1.10), all of these AP would be expected to yield HCHO after periodate oxidation. The C atom in HCHO would come from C-1 of these AP's. The ribose-AP is likely to be acyclic in nature and so would release a second HCHO from C-5 (compare to structures in fig 1.10). This could explain partly the higher values for glycation in fig 4.1. Fructose-derived AP will be discussed in chapter 5.

As an indication of sugar-induced damage in vivo, fluorescent-AGE might be better than the apparent AP. Protein fluorescence has been reported to increase during diabetes and ageing and has been related to the severity of diabetic complications (93). In vivo, glucose is believed to be the major sugar responsible for this fluorescence. The two recently characterised AGE namely pentosidine and pyrraline are both fluorescent in nature, and their levels have been reported to increase during diabetes (24, 28). Fluorescence generated by proteins upon incubation with sugars is due to buildup of AGE and not AP

since selective removal of AP by periodate treatment has been reported to have no effect on protein fluorescence (99).

In this study, incubation of BSA with the different sugars generated fluorescence in the order ribose >fructose >galactose >glucose ie similar to their expected rate of SB formation (fig 4.2). The rates of fluorescence generation is particularly high with fructose and ribose rapidly attaining maximum values within the first few days, followed by an apparent decline in the prolonged incubations possibly due to quenching. Other studies have shown that ribose induces faster fluorescence of lens crystallins than glucose (154), and so too does fructose with BSA (99), lens protein (100), collagen (101) and RNase (57). The order in which the four sugars react with BSA to generate fluorescence is similar to that with lysozyme (fig 4.4) and RNase (fig 4.5). It therefore seems that rate of fluorescent-AGE generated by proteins after reaction with different sugars will depend on the rate of SB formation irrespective of the protein used. For all three proteins fluorescence induced after ribation and fructation rapidly reaches saturation whereas the other sugars are probably aiming for saturation and therefore the sampling time may be important. For both lysozyme and RNase there is a lag phase for incubations in glucose and galactose. During this lag phase buildup of AP probably occurs giving rise to the increase in fluorescence seen after 7 and 5 days for lysozyme and RNase respectively.

I found that BSA is not a good model protein for crosslinking studies since it does not readily polymerise. BSA is probably more susceptible to intramolecular as opposed to intermolecular crosslinking. This is consistent with reports by Kato et al who too could not detect appreciable crosslinking of either BSA or HSA (155). In contrast, Sakurai et al, have suggested that

glycated HSA is a good model protein for crosslinking studies and have shown extensive polymerisation of HSA on 5% SDS gels (54). In this case however, the glycated fraction was separated from non-glycated HSA by affinity chromatography prior to electrophoresis. Glycated BSA does show reduced electrophoretic mobility which is particularly prominent with fructated BSA (fig 4.3). This reduced electrophoretic mobility has also been reported for glucated RNase (55), and is probably due to impaired SDS binding to proteins.

Since BSA is not a good model for crosslinking, I decided to investigate other proteins namely lysozyme and RNase. Dyer et al have shown that less than 1% of crosslinks in glucated lysozyme are fluorescent which means that the major AGE responsible for crosslinking is non-fluorescent and colourless (26). The pathways for crosslinked and non-crosslinked AGE may therefore operate independently of each other. To test this hypothesis, I compared crosslinking and fluorescence of ribated lysozyme and RNase glycated under identical conditions. I was able to show that ribated RNase generates fluorescence much faster than ribated lysozyme (fig 4.6). However, the ribated lysozyme (fig 4.7) crosslinked more readily than the RNase (fig 4.8 and 4.9). For this reason the effect of different sugars on crosslinking was undertaken to see how it relates to the fluorescence and AP formation rates.

Fructation-induced polymerisation of lysozyme is similar to that induced by ribation (table 4.1) and these results agree with those of Shin et al (18) who also showed that comparative rates of fructose and ribose-derived crosslinking of lysozyme were similar. This compares with the fluorescence studies where ribose-derived fluorescence was considerably higher for all proteins. A postulated intermediate of the Maillard reaction 3-DG is known to promote protein crosslinking in vitro, and it has been suggested (although not yet proven) that fructose may form this compound directly under physiological

conditions in addition to that formed via the Amadori route (155). This could account for the higher crosslinking by fructose. It therefore seems that in contrast to fluorophore formation, crosslinking may not necessarily depend on the initial rate of SB formation.

Although ribose rapidly induces crosslinking and fluorophore formation, its level in the plasma is well below 50uM (28), and probably derived from turnover of ribonucleic acids. Galactose has been reported to induce SB (11) and AP (153) formation faster than glucose. However, to my knowledge this is the first report to demonstrate faster formation of AGE by galactose compared to glucose. Galactataion has been well studied with respect to galactosaemia and at present there is no evidence that it contributes towards the undesirable effects in this disorder. Of these sugars fructose perhaps deserves the most attention since it can reach significant levels in vivo especially in diabetics. For this reason fructose has been selected and studied in depth in the remaining chapters of this thesis.

Fructose-Induced Glycation and AGE Formation

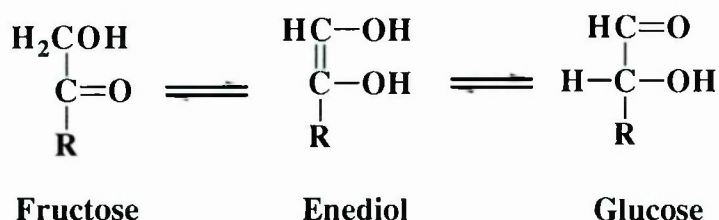
5.1: Introduction

In vitro studies have demonstrated that fructose is a more potent glycation agent than glucose (11) and upto 10 times more efficient at forming AGE (99). The possibility that fructose may contribute towards in vivo glycation has received little attention presumably due to its minute levels in the plasma. However, fructose is a permitted sweetener in diabetic foods and furthermore diabetics have been reported to have higher serum fructose levels compared to non-diabetic after ingestion of a fructose-rich diet (103). Increased activity of the sorbitol pathway in diabetes can lead to high levels of fructose in certain regions of the body notably those areas susceptible to secondary complications (107). In these regions fructose levels can increase by 22 fold and one study has reported levels upto 12 mM in the lens (108).

At present there is direct evidence for modification of lens protein (57) and indirect evidence for modification of Hb (104) and collagen (113) by fructose in vivo. In the previous chapter, apparent fructose-derived AP levels were lower than those derived from glucose when measured by the periodate method and the possible reasons for this have been discussed in chapter 4. Most assays are designed to detect the glucose-AP and since the fructose-derived AP are different in structure they may not be detected by the various assays used to quantify glycation.

McPherson et al (57) have shown that approximately 85% of fructose-derived AP is attached to the protein via C-2 (fig 5.1). Fructose-derived AP attached to protein via C-2 may be theoretically an aldose (F-AP 1) or ketose (F-AP 2) although this has yet to be proved (99) (see fig 5.1). However this group also

demonstrated that the remaining 15% of AP was identical to that formed by glucose (fig 1.1). This small amount of glucose-AP is probably formed from fructose since the two sugars have a common enediol ie



From theoretical considerations it would appear that other assays may also underestimate fructation. Assays commonly used in routine laboratories to measure glycation of serum proteins and Hb include the fructosamine and phenylboronate affinity chromatography methods respectively. These assays together with the TBA assay, often used to measure glycated collagen, were investigated to see whether they too underestimate fructation.

A common feature of all AP whether derived from glucose or fructose is the presence of a carbonyl group and an assay which can quantify this may provide a true estimate of fructation. The dinitrophenylhydrazine (DNPH) procedure used to assay free carbonyl groups has been used in earlier studies to quantify glycation of Hb (156) but was discontinued on the basis that a very small percentage of the glucose-derived AP exists in the acyclic form. However, in more recent studies this method has been successfully used to assay for glucation of albumin (157) and to quantify the reaction between glyceraldehyde and Hb (158). In this work an attempt was made to use this assay to estimate in vitro fructation of albumin.

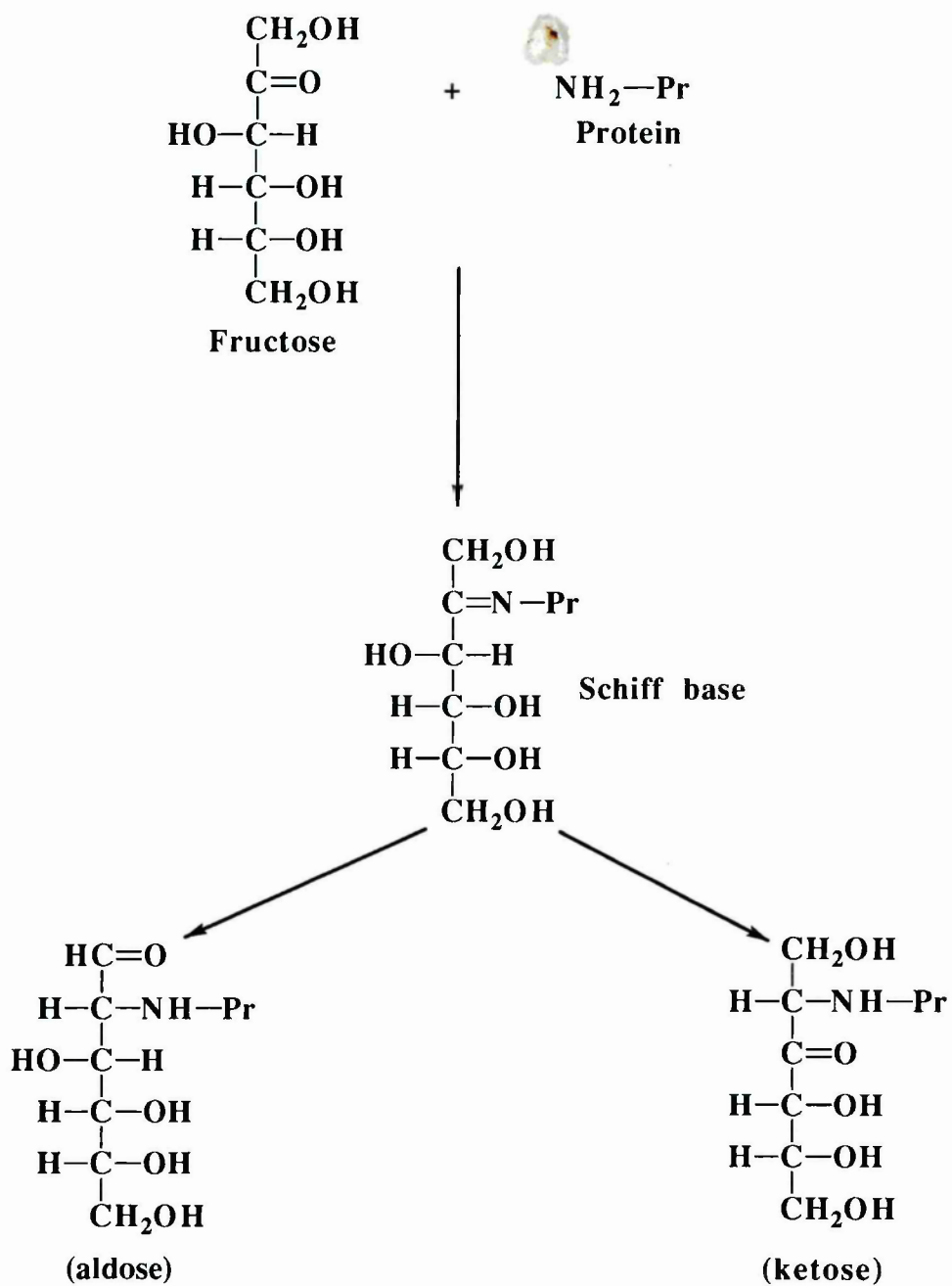


Fig 5.1: Fructose derived Amadori products (adapted from ref (99))

5.2: Aims

- 1: To compare the rates of fluorescence generation after in vitro glucation and fructation using BSA as a model protein.
- 2: To compare the rates of glucose- and fructose-derived crosslinking using lysozyme as a model protein.
- 3: Extent of fructation of BSA by the TBA, fructosamine and phenylboronate affinity chromatography methods was measured and compared to the values for glucated BSA in order to assess whether these assays like the periodate method underestimate fructation.
- 4: The DNPH assay detects free carbonyl groups and should detect all fructose-derived AP therefore reflecting the true extent of fructation. This assay was used to measure and compare glucation and fructation of BSA.

5.3: Experimental Protocol

In Vitro Glycation: BSA fatty acid free (10mg/ml) was incubated in 0.5M sugar at 37°C for upto 24 days in 0.05M sodium phosphate buffer/3mM sodium azide, pH 7.4 (section 2.1). Aliquots were withdrawn after various time intervals and dialysed exhaustively against distilled water or column starting buffer (for the phenylboronate affinity chromatography assay) (section 2.2).

Protein Concentration: This was assayed using the Pierce BCA protein assay reagent (section 2.4.2).

Measurement of Glycation: AP levels were measured using periodate microassay (section 2.8.2), TBA microassay (section 2.8.3), phenylboronate affinity (PBA) chromatography (section 2.8.5), serum fructosamine (SFA) assay (section 2.8.6) and DNPH microassay (section 2.8.4).

Measurement of AGE: AGE were measured by their crosslinking on SDS gels (section 2.9.2) or their fluorescence changes (section 2.9.1).

5.4: Results

Fig 5.2 shows the effects of in vitro glucation and fructation on fluorescence of serum albumin and clearly suggests that fructose has a greater effect on protein structure than glucose. Using lysozyme as a model protein for crosslinking, a similar result was obtained, with fructose-induced crosslinking being greater than that induced by glucose (figs 5.3 and 5.4).

However, glycation assays would suggest the opposite (table 5.1). Glucose appears to produce some 3-10 times more AP than fructose, at each stage of the in vitro glycation.

Values for fructation measured by the TBA and fructosamine assay are less than 15% of those for glucation. In contrast fructose-derived AP levels measured by periodate and phenylboronate assays are between 30-40% of those for glucose-derived AP. Fig 5.5 shows moles of carbonyl groups per mole of protein formed by fructated and glucated BSA over a period of 16 days. The values for fructation are approximately 10 times higher than those for glucation. These experiments were repeated 3 times and the most representative results are shown.

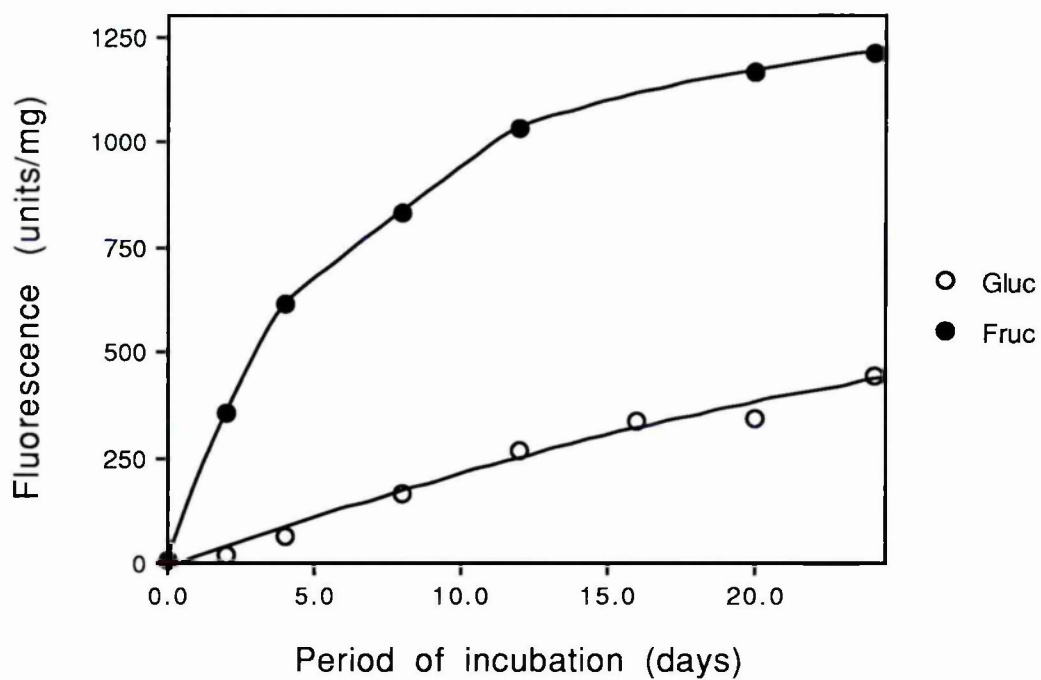


Fig 5.2: Fluorescence of BSA (10mg/ml) incubated in 0.5M glucose or fructose

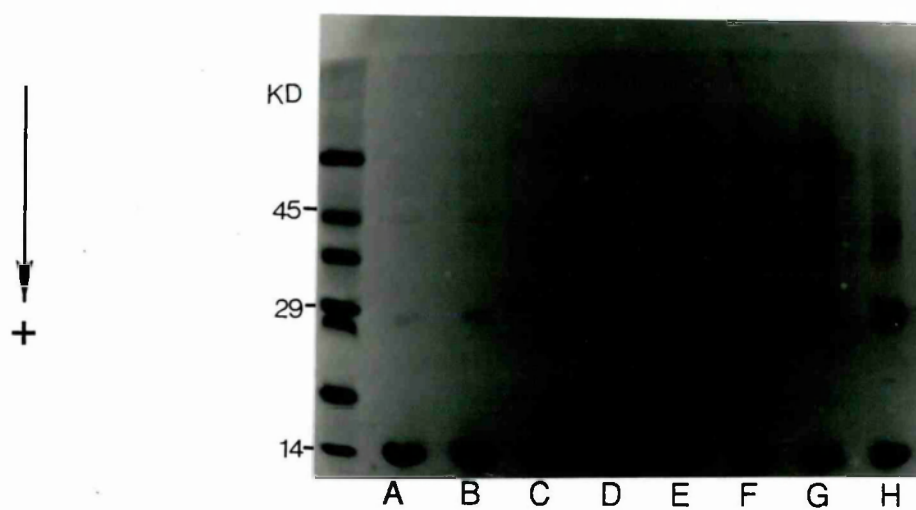


Fig 5.3: SDS-PAGE of lysozyme (10mg/ml) glycated in vitro by 0.5M glucose for 0, 2, 4 and 6 days (lanes A, B, C and D respectively) or 0.5M fructose for 0, 2, 4 and 6 days (lanes E, F, G and H respectively).

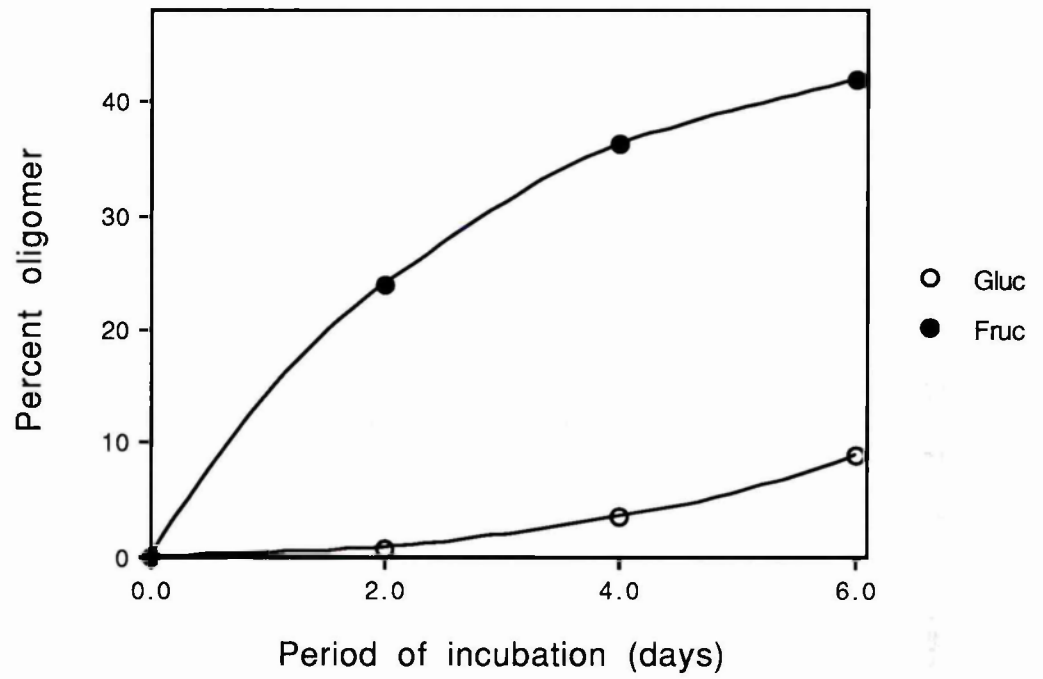


Fig 5.4: Crosslinking of lysozyme (10mg/ml) incubated in 0.5M glucose or fructose

Time Period (days)	Periodate (moles/mole)		TBA (HMF/mg)		SFA (mmol/l)		PBA (%)	
	G-BSA	F-BSA	G-BSA	F-BSA	G-BSA	F-BSA	G-BSA	F-BSA
0	0.42	0.42	5.64	5.20	0.40	0.60	9.30	9.80
5	3.53	1.20	45.80	6.55	4.40	0.80	88.0	29.0
10	3.88	1.34	61.20	7.42	6.30	0.70	94.0	34.0
16	4.92	1.62	72.03	9.68	8.40	0.90	93.0	37.0

Table 5.1: Apparent AP levels measured by different assays for BSA (10mg/ml) glycated in vitro by 0.5M glucose (G-BSA) or fructose (F-BSA) for upto 16 days

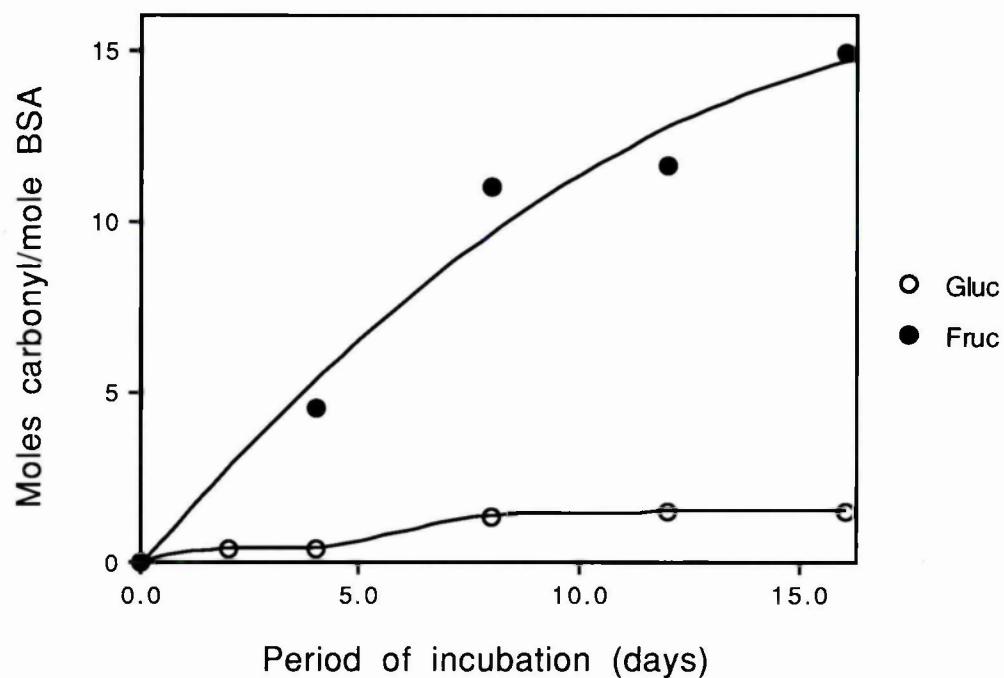


Fig 5.5: Protein bound carbonyl groups measured by the DNPH microassay for BSA (10mg/ml) glycosylated by 0.5M glucose or fructose.

5.5: Discussion

Fructose-induced changes to protein structure occur much faster than those induced by glucose. Using BSA as a model protein, I have demonstrated that fructose forms fluorescent-AGE considerably faster than glucose in vitro (fig 5.2). This finding is consistent with other studies for BSA (99) and indeed other proteins ie RNase (57) and collagen (101). Similarly fructose-derived crosslinks form faster than those induced by glucose and this was demonstrated using lysozyme as a model protein (fig 5.3). A possible reason for this fast AGE formation may be that the fructose-derived aldehydic AP (F-AP 1) is particularly reactive and participates in post-Amadori reactions faster than those derived from glucose. Dicarbonyl compounds such as 3-deoxyglucosones (3-DG) derived from AP have been shown to induce both crosslinking and fluorophore formation of proteins in vitro (17, 18). Fructated proteins can form twice as much 3-DG as those that have been glucated (155), and furthermore fructose is known to form 3-DG directly at high temperatures (159) and it has been suggested that this may occur even under physiological conditions (155).

A higher proportion of fructose (0.7%) exists in the acyclic form compared to glucose (0.002%) and so fructose would be expected to form SB 350 times faster than glucose (10). Aldoses are approximately 50 times more reactive than ketoses, and so taking both factors into account, fructose would be expected to react 7.5 times faster than glucose. Indeed this is consistent with the in vitro studies of Bunn et al using radiolabelled sugars (11). Assuming that the rate of Amadori rearrangement is approximately the same for glucose and fructose-derived AP (although there is no information on this), then fructose-derived AP would be expected to form faster than those from glucose. However, this is contrary to the results in table 5.1. There are two possible

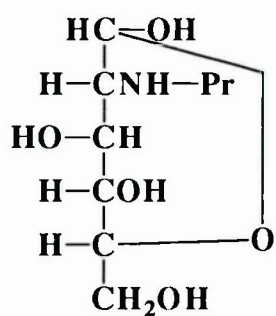
explanations for this and it is likely that both are responsible for the apparent low levels of AP formed by fructated proteins. First since fructose-derived AP are rapidly converted to AGE, there is little buildup of these AP and this may account for their lower levels. The second reason may be that most assays are designed to detect the glucose-derived AP which is a ketone where the carbonyl group is on C-2 and is attached to protein via C-1. In contrast the fructose-derived aldehydic isomer (F-AP 1) is not expected to liberate HCHO after periodate oxidation (ref (99), see fig 5.6) nor is it expected to cyclize to form HMF and so may not be detected by either periodate or TBA assays. In addition, aldehydic AP's do not readily dehydrogenate (160) and so may not react with redox dyes such as NBT in the serum fructosamine assay. Furthermore, the conformation of this aldehydic AP lacks the cis diols needed for strong adsorption in the phenylboronate affinity chromatography method and hence a high percentage glycation reading. This could be because sugars may adopt a fructose-like conformation in the glucose-AP (ie with a furanose ring as shown in the penultimate cyclic structure of fig 1.1), or a pyranosyl structure in the fructose-AP (as shown in the first cyclic structure of fig 1.1 but with C-1 substituted by -NHR). Fructose is well known to bind more strongly to phenylboronate resins than glucose does (161, 162). The ketone isomer with protein attached to C-2 (F-AP 2) liberates HCHO upon periodate oxidation (fig 5.6) but is unlikely to cyclize and form HMF, which may account for the higher fructation values obtained with the periodate than TBA assay. The 15% of fructose-derived AP identical to that from glucose probably accounts for the small positive reactions in the TBA and serum fructosamine assay.

From table 5.1, it can be seen that the major assays used to quantify glycation may underestimate fructation. However, it is clear from the fluorescence and

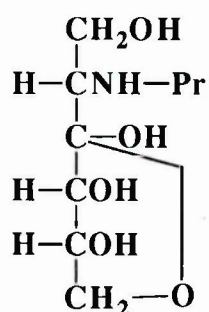
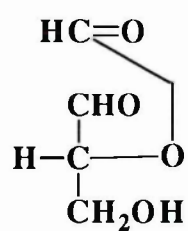
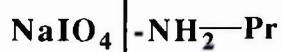
crosslinking studies that fructose-induced changes to protein structure occur much faster than those induced by glucose. Evidence for in vivo fructation has been obtained only by charge based glycation assays possible with Hb (104), which would detect all the fructose-derived AP. Charge based separations depend on a drop in pK when amino groups are converted to SB or to AP, and this occurs for all AP during fructation. In subjects monitored only by the phenylboronate or serum fructosamine assays on serum albumin, any in vivo fructation would be greatly underestimated. Therefore dietary fructose could be causing structural damage that at present goes undetected. This could be relevant clinically, since diabetics can convert glucose to fructose via the sorbitol pathway and dietary fructose is a permitted sweetener for diabetics and a major component of the non-diabetic diet (eg combined with equimolar quantities of glucose in sucrose).

The DNPH assay was first used by Fields and Dixons to detect protein bound carbonyl groups (145). Studies by Ghiggeri et al have shown that with albumin a small percentage of ketoamine groups (~ 15%) generate colour as the phenylhydrazone, and borohydride reduction prior to assay results in no colour yield (157). Any SB, free sugar or 3-DG will result in the formation of hydrazones of low molecular weight (157), but extensive dialysis prior to assay should exclude any interference by these. It is important to stress that the DNPH assay should detect both aldehydic and ketonic carbonyl groups and would be expected to detect all fructose-derived AP. If a higher proportion of fructose-derived AP exist in the acyclic form (although there is no information on this) this may account for the higher levels of DNPH derivative produced by fructated compared to the glucated BSA. Furthermore carbonyl groups from AGE may also be detected and indeed the fluorescent-AGE pyrraline

possesses a free carbonyl group and so too may others. This possibility becomes more plausible when comparing the increase in protein bound carbonyl groups (fig 5.5) for glucated and fructated BSA with their corresponding fluorescence changes (fig 5.2). In either case, the DNPH reaction provides a colorimetric assay that gives a truer measure of fructose-induced changes to protein structure than any of the other assays.



Fructose-AP
(aldose)



Fructose-AP
(ketose)

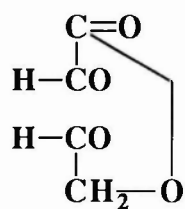
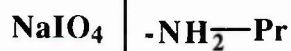


Fig 5.6: Periodate oxidation of fructose-AP (adapted from ref (99)).

CHAPTER 6

Effect of Phosphate, pH and Calcium on AGE Formation

6.1: Introduction

Glycation has been shown to increase with period of incubation, concentration of sugar, and temperature (41). The extent of glycation can also be affected by the pH and concentration of phosphate.

Studies on the effects of pH and phosphate concentrations have looked at changes in AP formation and not post-Amadori products. When the pH is increased from 4.5 to 9.0, the extent of glycation increases (41). At a higher pH, a smaller proportion of the ϵ -amino groups on lysine residues will be charged (ie less dissociation) and hence greater reactivity. Furthermore, a rise in pH can affect the percentage sugar in acyclic form ie as pH of glucose solution is increased from 6.5 to 7.5, the percentage sugar in the acyclic form increases from 0.012% to 0.04% (163). Phosphate has been shown to increase extent of AP formation by catalysing the Amadori rearrangement (164), and has no effect on the extent of SB formation. Phosphate could also increase reactivity of sugar since it increases the proportion of sugar in the acyclic form (165). Normal plasma levels of phosphate are between 1.6 to 2.3mM.

Mineral ions such as FeSO_4 , CuSO_4 and CaCl_2 in browning reactions have been studied by food chemists at elevated temperatures, however, their effects are controversial. For eg CaCl_2 has been reported to reduce browning by Smith and Cline (166) and Yu et al (167) whereas Fry and Stegink reported an increase in this reaction (168). More recent work by Labuza has suggested that electrolytes have no effect on the browning reaction (169). Calcium ions have

been reported to shift equilibrium in favour of the α -anomer, but their effect on proportion of sugar in acyclic form is not known (170). Plasma levels of calcium ions are about 2.5mM and during hypercalcaemia can rise to 5mM. Approximately 0.9mM calcium is bound to albumin whereas 1.6mM exists as free ionised calcium.

In this study the effect of phosphate, pH and calcium ions on the formation of glucose-derived fluorescent-AGE were studied. Almost all studies so far have investigated the effects of these factors on glucose-induced changes and since the mechanism for fructose-induced changes to protein structure are different, the effect of phosphate, pH and calcium ions on fructose-derived fluorescent-AGE were also investigated.

Eble et al (55) and McPherson et al (57) have reported increased crosslinking and fluorescence of glycated RNase upon reincubation after removal of sugar. This rise in AGE was attributed to decreased competition between Amadori carbonyl groups and sugar carbonyl groups for free amino groups from protein. This led to the suggestion that once glycated, a protein will participate in AGE formation even on return to normoglycaemia, explaining why certain diabetic complications progress even after strict glycaemic control (171). In contrast, Schleicher et al found no significant increase in fluorescence generation of glycated albumin upon reincubation after exhaustive removal of sugars. However, a small consumption of Amadori groups was noted (90). Similarly, Kato et al did not observe a significant rise in browning of ovalbumin upon reincubation after removal of unbound sugar (172). A study by Rucklidge et al have found no evidence for a reaction between fructosyllysine (glucose-derived AP) and free amino groups from lysine residues in the absence of free sugar (173).

In this chapter, glucated and fructated BSA were reincubated in the absence of sugars and the formation of fluorescent-AGE monitored. To examine whether the effect of phosphate, pH and calcium ions on fluorescence changes are due to effects on post- as opposed to pre-Amadori reactions, the effect of these factors on fluorescent-AGE in absence of sugars were also investigated. Since many of the crosslinked-AGE are non-fluorescent, the effect of phosphate, calcium ions and pH on formation of crosslinked-AGE upon reincubation of glycated lysozyme, after removal of sugars, was also investigated.

6.2: Aims

- 1: To investigate the effect of phosphate, calcium ions and pH on generation of fluorescent-AGE formed by BSA incubated in the presence of glucose or fructose.
- 2: To investigate the effect of phosphate, calcium ions and pH on post-Amadori reactions responsible for fluorescent-AGE by incubating the isolated AP ie glucated or fructated BSA in absence of sugars.
- 3: To investigate the effect of phosphate, calcium ions and pH on post-Amadori reactions responsible for crosslinked-AGE by incubating glucated or fructated lysozyme in the absence of sugars.

6.3: Experimental Protocol

In Vitro Glycation: Fast glycation involved incubating BSA (fraction V) at 10mg/ml in 0.5M glucose or fructose at 37°C in either (a) 0.05M HEPES/3mM sodium azide, pH 7.4 +/- 2.5-10mM calcium chloride or (b) in 0.05M sodium phosphate buffer/3mM sodium azide, pH 5.7-8.0, or (c) 0.05-0.3M sodium phosphate/3mM sodium azide, pH 7.4 for upto 21 days (section 2.1).

Glycated protein for reincubation was prepared by incubating BSA (12mg/ml) in 0.5M glucose or fructose for 16 and 11 days respectively in 0.05M sodium phosphate/3mM sodium azide buffer, pH 7.4 at 37°C. Lysozyme was also glycated by glucose and fructose for 6 and 4 days respectively under the above conditions. All proteins were dialysed exhaustively against water to remove unbound sugars (section 2.2). BSA (6mg/ml) was incubated at 37°C alone and in 0.5M glucose for 16 days and in 0.5M fructose for 11 days in 0.05M sodium phosphate buffer/3mM sodium azide, pH 7.4. The fluorescence was determined before and after 4 days of dialysis with 8 changes.

Reincubation of Glycated Proteins: Glycated proteins were reincubated for the stated periods of time at 37°C in; (a) calcium (2.5-10mM) in HEPES buffer, pH 7.4 (b) in 0.05M sodium phosphate buffer of varying pH (5.7-8.0) or (c) sodium phosphate buffer of varying concentration (0-0.2M). In all cases the buffers contained 3mM sodium azide to prevent bacterial growth. Samples were dialysed against distilled water (section 2.2) and stored frozen.

Protein Concentration: This was determined using the Pierce protein assay reagent (section 2.4.2).

Measurement of AGE: AGE were measured by their fluorescence changes (section 2.9.1) or crosslinking on SDS gels (section 2.9.2).

Statistical analysis was performed using the Students t test.

6.4: Results

Effect of phosphate: Phosphate (0-0.2M) has no effect on fluorescence of BSA incubated in the absence of sugars (fig 6.1). However, a dose dependent effect of phosphate is demonstrable on fluorescence of BSA incubated in glucose (fig 6.2) or fructose (fig 6.3), and is more pronounced for the latter. Phosphate promotes a rapid increase in fructose-induced fluorescence

particularly during the first 5 days of incubation but then slows down. In contrast, the rise in fluorescence of BSA incubated in glucose is gradual but linear throughout the period of incubation. This would be expected since the absolute extent of glycation here is still well below the saturation levels approached by fructose.

To study the effect of phosphate on post-Amadori reactions only, glycated protein prepared in vitro was dialysed to remove unbound sugar and SB and then reincubated for a certain period of time. On reincubation of dialysed glucated BSA in a non-phosphate (HEPES) buffer (fig 6.4) in the absence of sugar, no further increase in fluorescence is detectable. However, some further fluorescence is generated upon reincubation in a phosphate buffer and this effect is higher with increasing concentrations of phosphate (fig 6.4). With fructated BSA, virtually no further rise in fluorescence is detectable in the absence of sugar under these conditions and phosphate appears to have no effect on post-Amadori reactions responsible for fluorescence generation (fig 6.5).

Lysozyme was chosen as a model protein to study crosslinked-AGE since it readily polymerises. Non glycated lysozyme, glucated and fructated lysozyme showed no increase in crosslinking upon reincubation in the absence of sugars for 9 days and phosphate appeared to have a small inhibitory effect on crosslinking of both glucated and fructated lysozyme (fig 6.6 and table 6.1).

Effect of pH: An increase in pH (from 5.7 to 8.0) has no effect on the fluorescence of BSA incubated alone (fig 6.7), but does increase the fluorescence of BSA incubated in glucose (fig 6.8) or fructose (fig 6.9). The fluorescence of glucated BSA is approximately 8 times higher at pH 8 compared to pH 5.7 after 21 days of incubation (fig 6.8). With fructated BSA the

increase reaches saturation with maximum values being attained after 7 days of incubation (fig 6.9). As with the phosphate studies, reincubated glucated BSA generated a small increase in fluorescence which increased with pH (fig 6.10) but no change in fluorescence of fructated BSA was detectable even at high pH (fig 6.11). As the pH was increased from 5.7 to 8.0, the crosslinking of glucated but not fructated lysozyme increased twofold after 9 days of reincubation in the absence of sugar (fig 6.12 and table 6.2).

Effect of calcium: Calcium ions have no effect on fluorescence of BSA incubated alone (fig 6.13), but do increase the fluorescence of BSA incubated in glucose (fig 6.14) or fructose (fig 6.15) although this increase is not as dramatic as with the phosphate and pH studies. Glucated BSA (fig 6.16) and fructated BSA (fig 6.17) reincubated in the absence of sugar in HEPES buffer shows no increase in fluorescence and calcium ions have no effect in either case. Similarly, calcium ions have no effect on the crosslinking of lysozyme, glucated and fructated lysozyme reincubated in the absence of sugars for upto 9 days (fig 6.18 and table 6.3).

These experiments were repeated two times ($n=2$) and the most representative results are shown.

Fig 6.19 shows the fluorescence of BSA and glycated BSA before and after dialysis. There is a significant rise in fluorescence of both glucated ($p < 0.05$) and fructated BSA ($p < 0.001$).

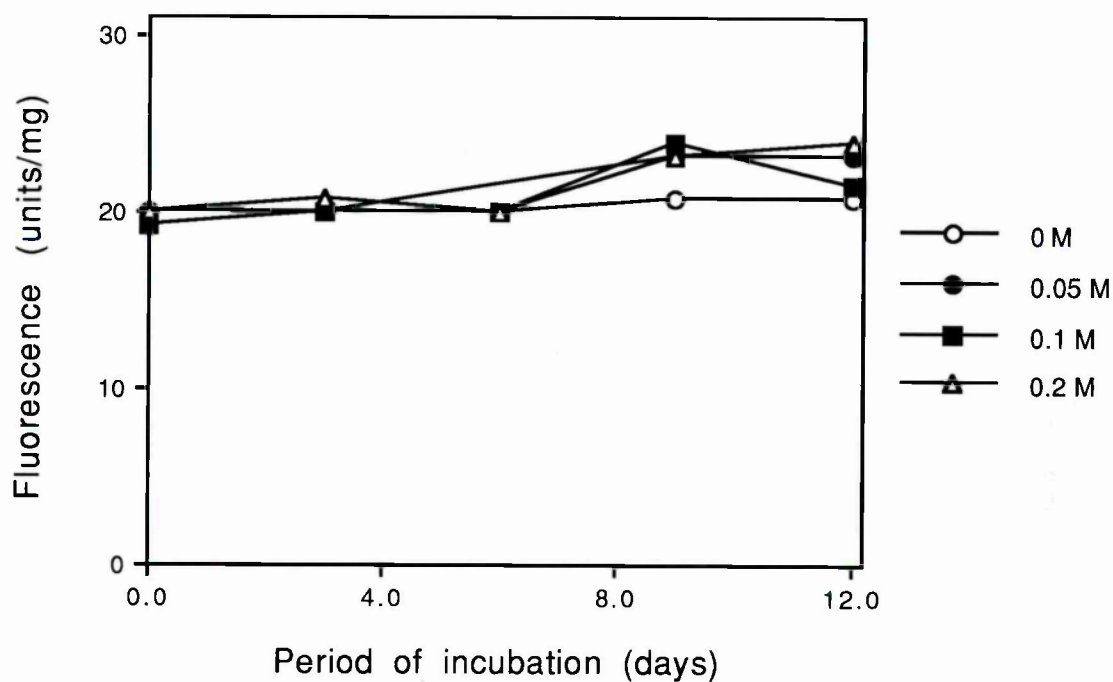


Fig 6.1: Effect of phosphate on fluorescence of BSA (10mg/ml) incubated in the absence of sugar

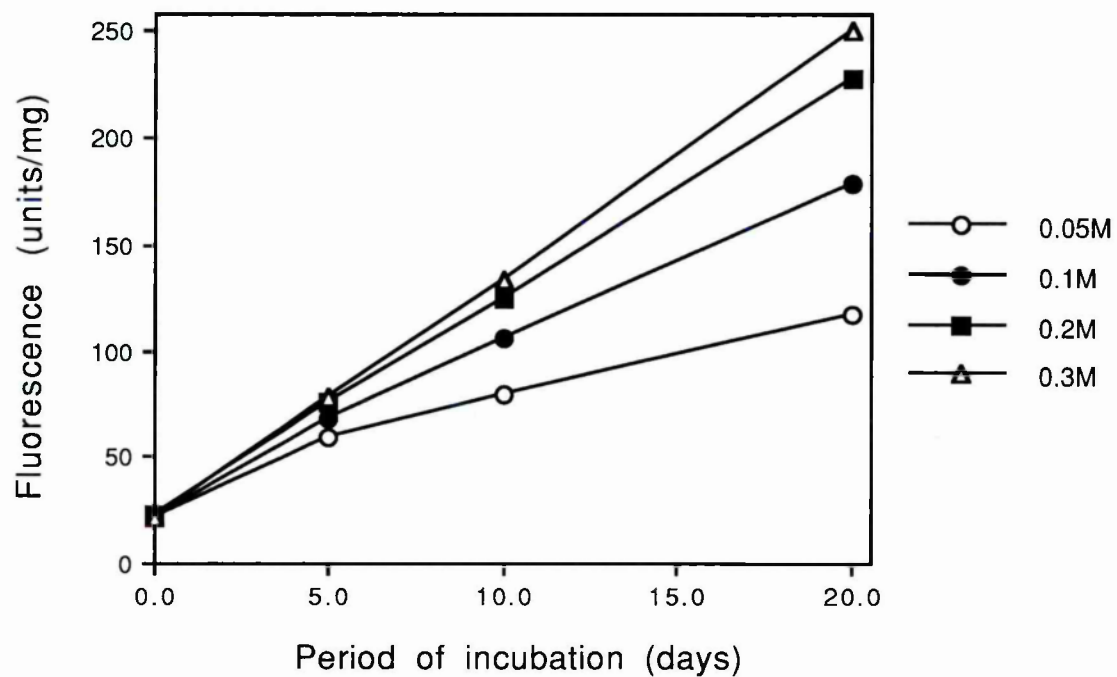


Fig 6.2: Effect of phosphate on fluorescence of BSA (10mg/ml) incubated in 0.5M glucose

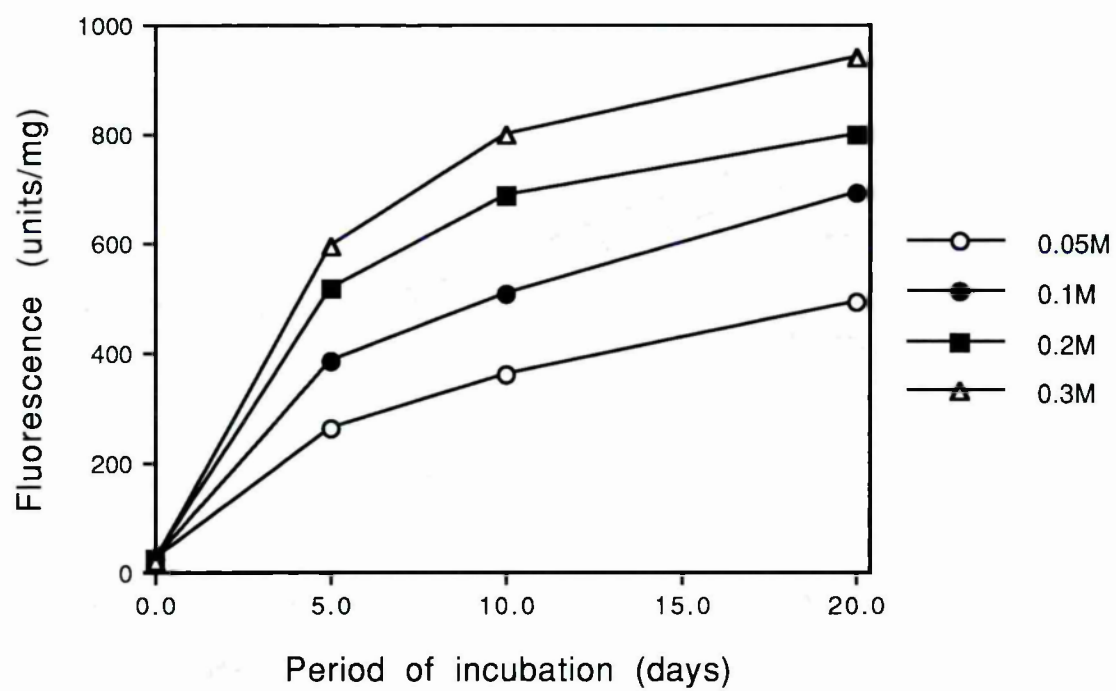


Fig 6.3: Effect of phosphate on fluorescence of BSA (10mg/ml) incubated in 0.5M fructose

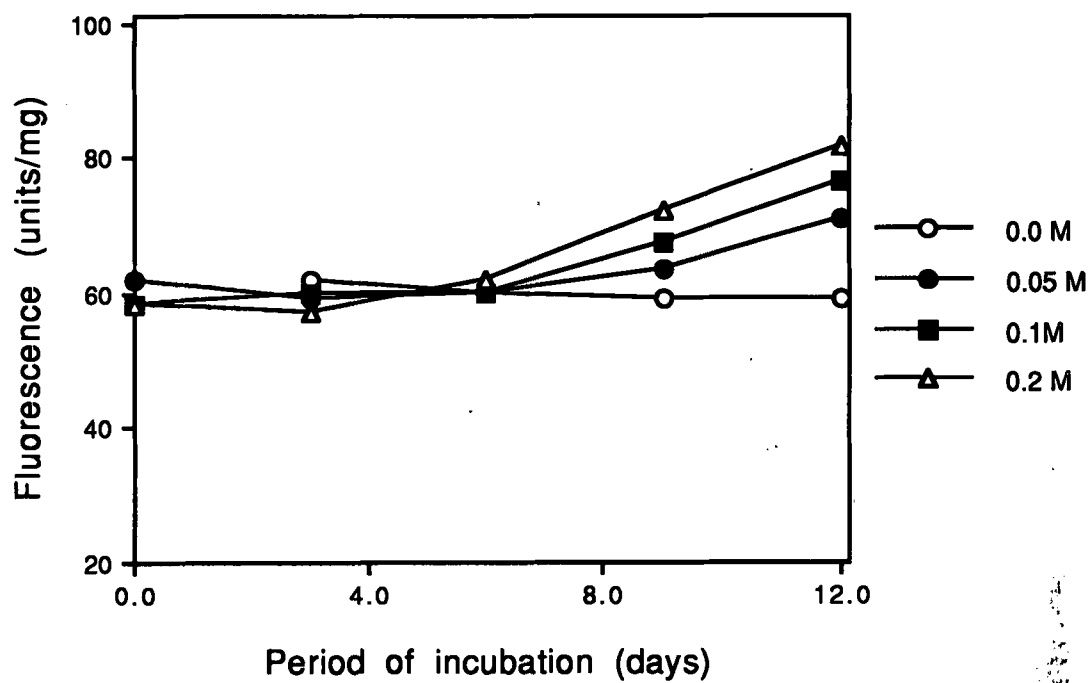


Fig 6.4: Effect of phosphate on fluorescence of glucated BSA reincubated in the absence of sugar

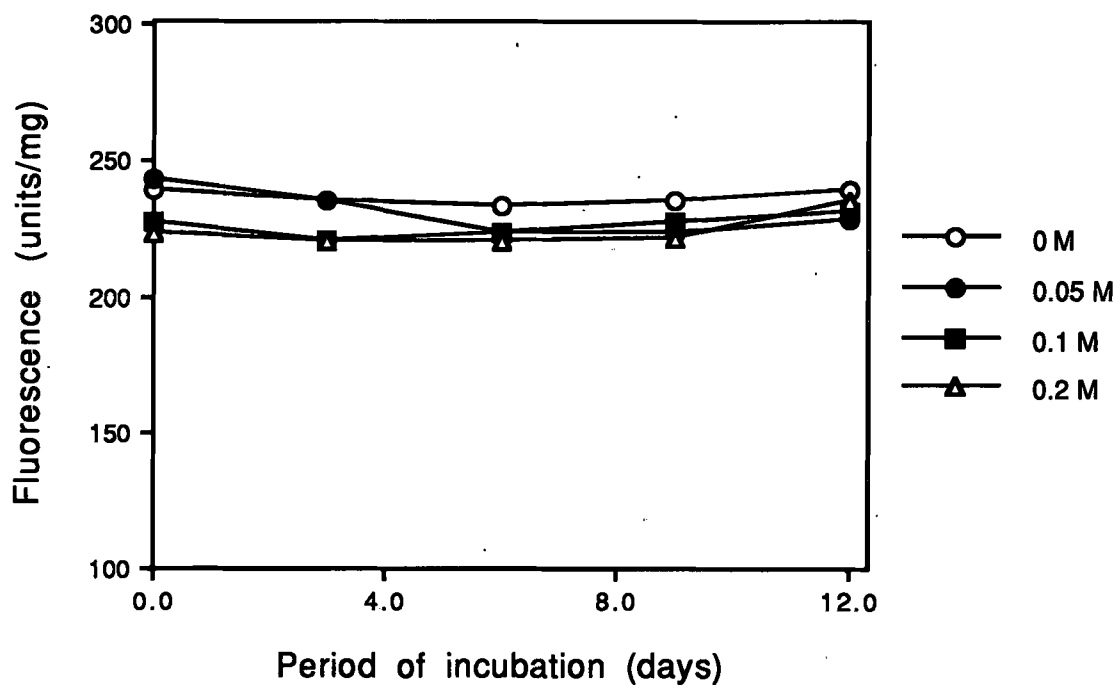


Fig 6.5: Effect of phosphate on fluorescence of fructated BSA reincubated in the absence of sugar

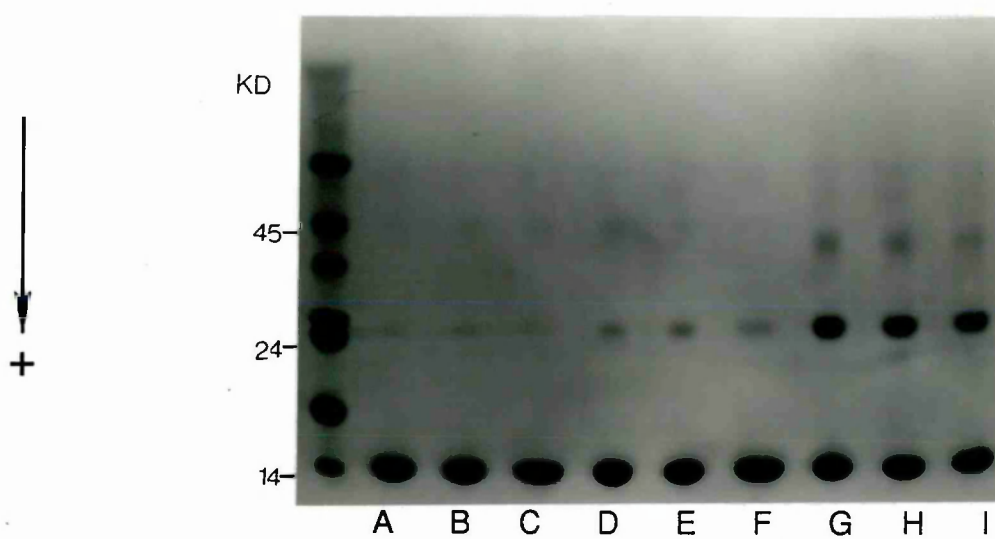


Fig 6.6: SDS-PAGE of lysozyme (lanes B and C), glucated lysozyme (lanes E and F) and fructated lysozyme (lanes H and I) incubated in 0.05 and 0.2M phosphate respectively for 9 days in the absence of sugar. Also shown is lysozyme (lane A), glucated lysozyme (lane D) and fructated lysozyme (lane G) before reincubation.

Protein sample	Time (days)	Phosphate (M)	% Oligomer
Lysozyme	0	0.05	0
Lysozyme	9	0.05	0
Lysozyme	9	0.20	0
Gluc lysozyme	0	0.05	6.4
Gluc lysozyme	9	0.05	6.5
Gluc lysozyme	9	0.20	5.7
Fruc lysozyme	0	0.05	28.3
Fruc lysozyme	9	0.05	28.0
Fruc lysozyme	9	0.20	24.7

Table 6.1: Effect of phosphate on crosslinking of lysozyme, glucated lysozyme and fructated lysozyme reincubated in the absence of sugar

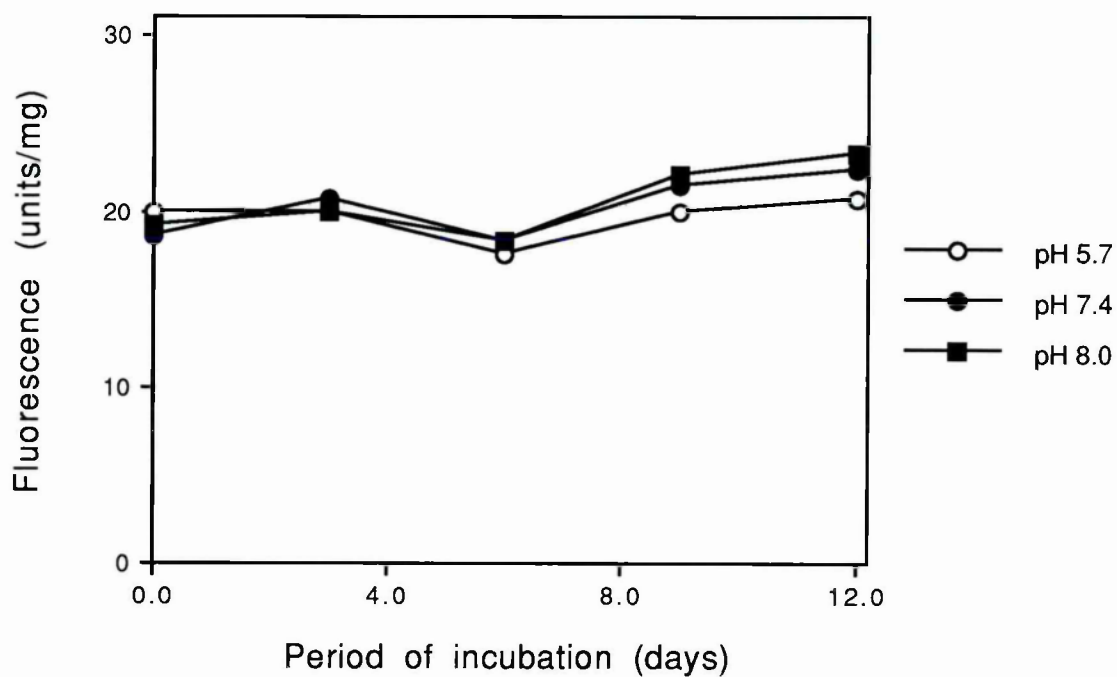


Fig 6.7: Effect of pH on fluorescence of BSA (10mg/ml) incubated in the absence of sugar

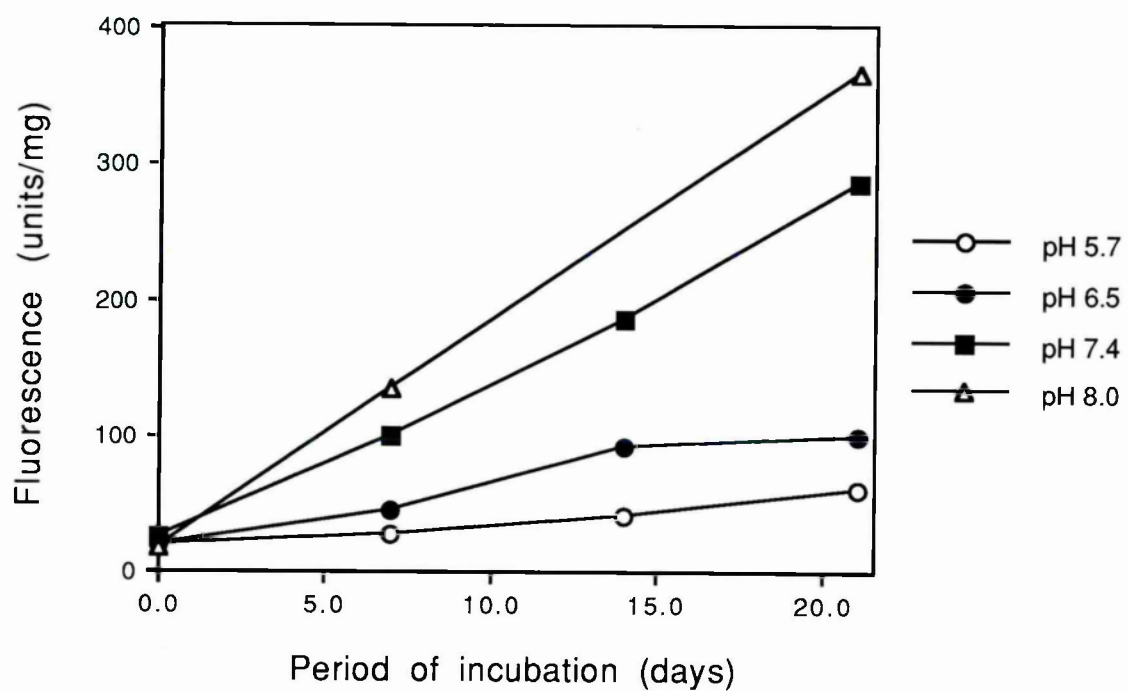


Fig 6.8: Effect of pH on fluorescence of BSA (10mg/ml) incubated in 0.5M glucose

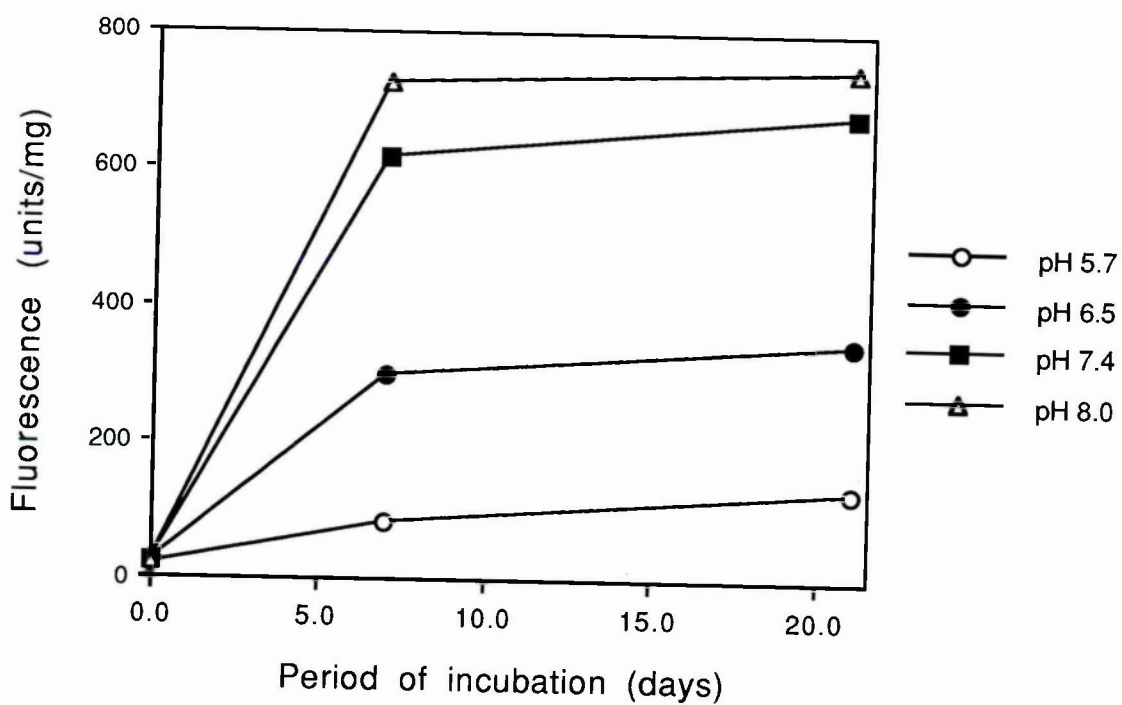


Fig 6.9: Effect of pH on fluorescence of BSA (10mg/ml) incubated in 0.5M fructose

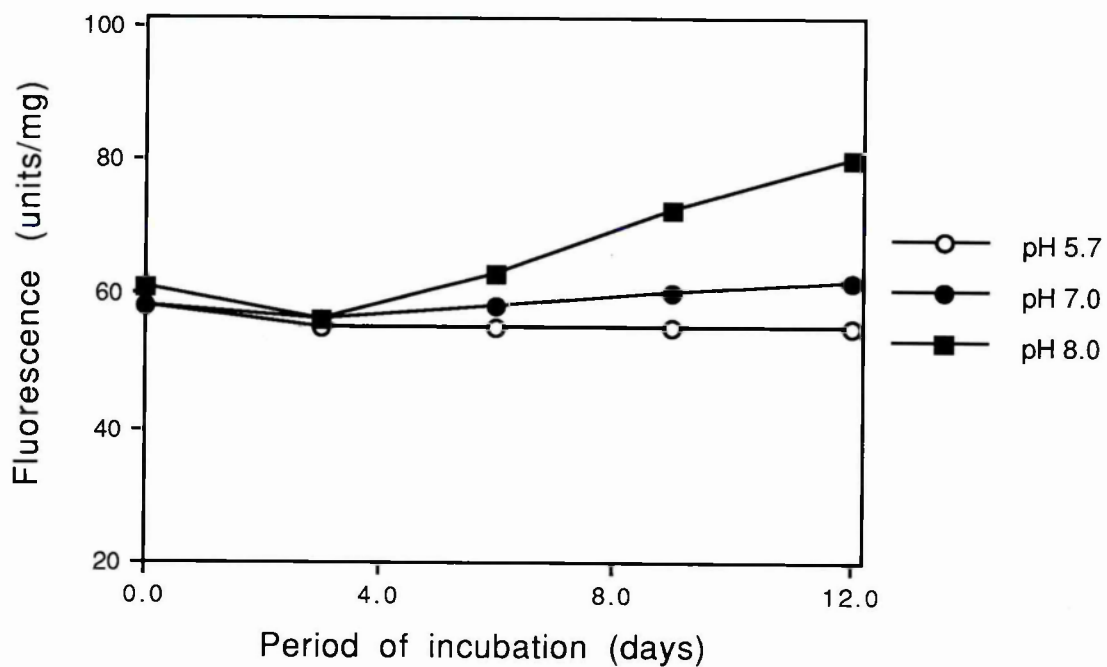


Fig 6.10: Effect of pH on fluorescence of glucated BSA reincubated in the absence of sugar

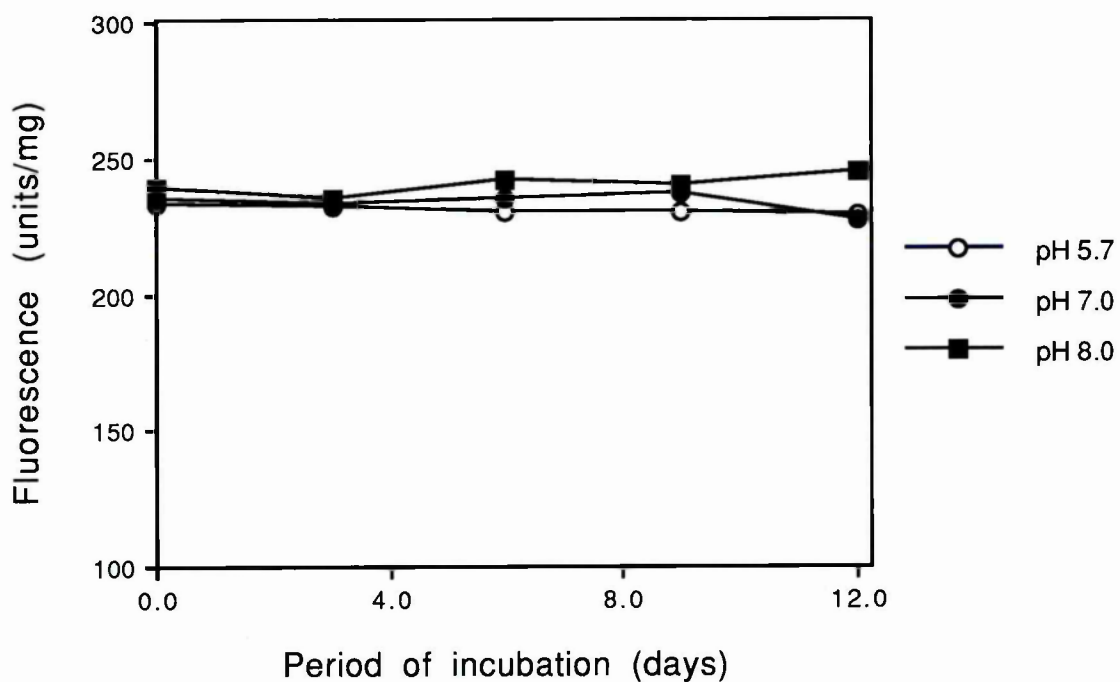


Fig 6.11: Effect of pH on fluorescence of fructated BSA reincubated in the absence of sugar

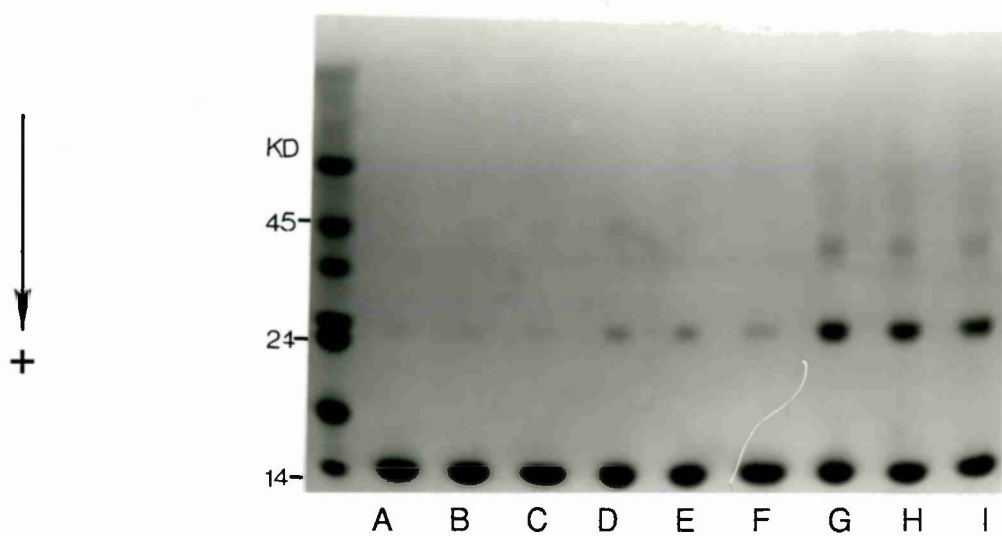


Fig 6.12: SDS-PAGE of lysozyme (lanes B and C), glucated lysozyme (lanes E and F) and fructated lysozyme (lanes H and I) incubated in buffer of pH 5.7 and 8.0 respectively for 9 days in the absence of sugar. Also shown is lysozyme (lane A), glucated lysozyme (lane D) and fructated lysozyme (lane G) before reincubation.

Protein sample	Time (days)	pH	% Oligomer
Lysozyme	0	5.7	0
Lysozyme	9	5.7	0
Lysozyme	9	8.0	0.2
Gluc lysozyme	0	5.7	3.6
Gluc lysozyme	9	5.7	3.2
Gluc lysozyme	9	8.0	6.4
Fruc lysozyme	0	5.7	28.0
Fruc lysozyme	9	5.7	28.5
Fruc lysozyme	9	8.0	25.6

Table 6.2: Effect of pH on crosslinking of lysozyme, glucated lysozyme and fructated lysozyme reincubated in the absence of sugar

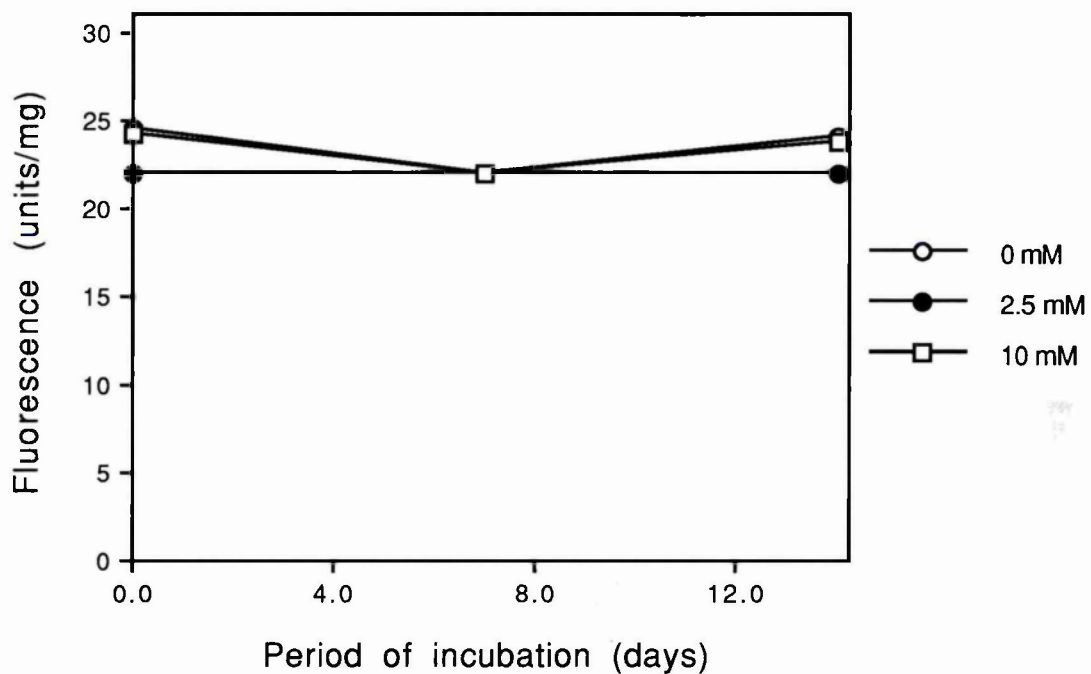


Fig 6.13: Effect of calcium on fluorescence of BSA (10mg/ml) incubated in the absence of sugar

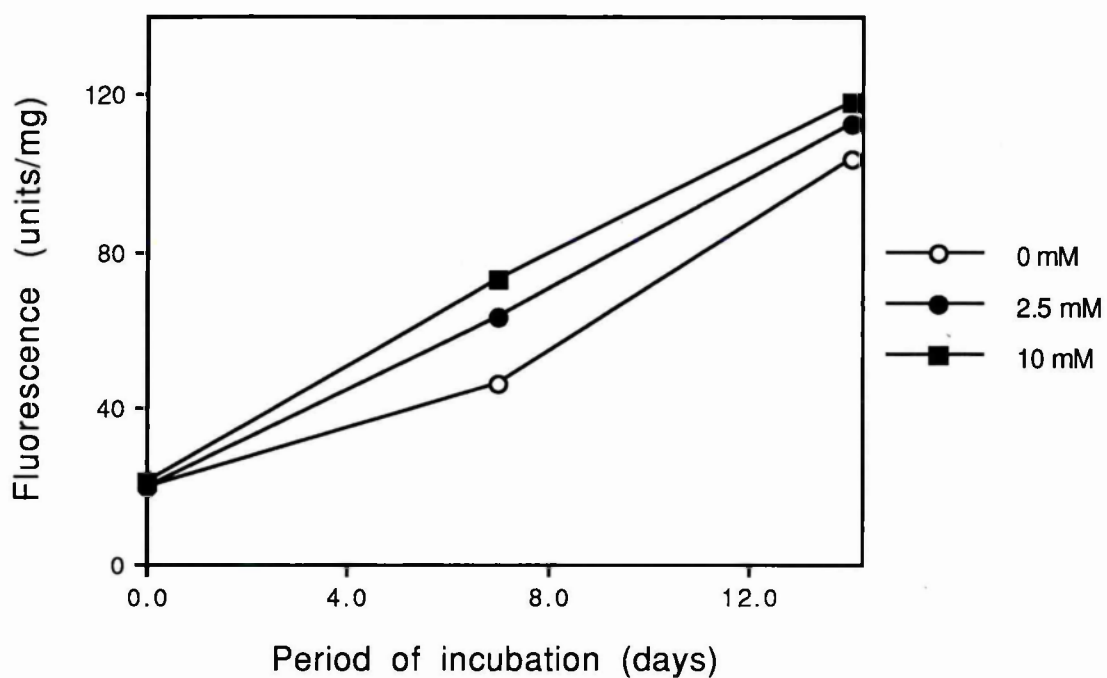


Fig 6.14: Effect of calcium on fluorescence of BSA (10mg/ml) incubated in 0.5M glucose

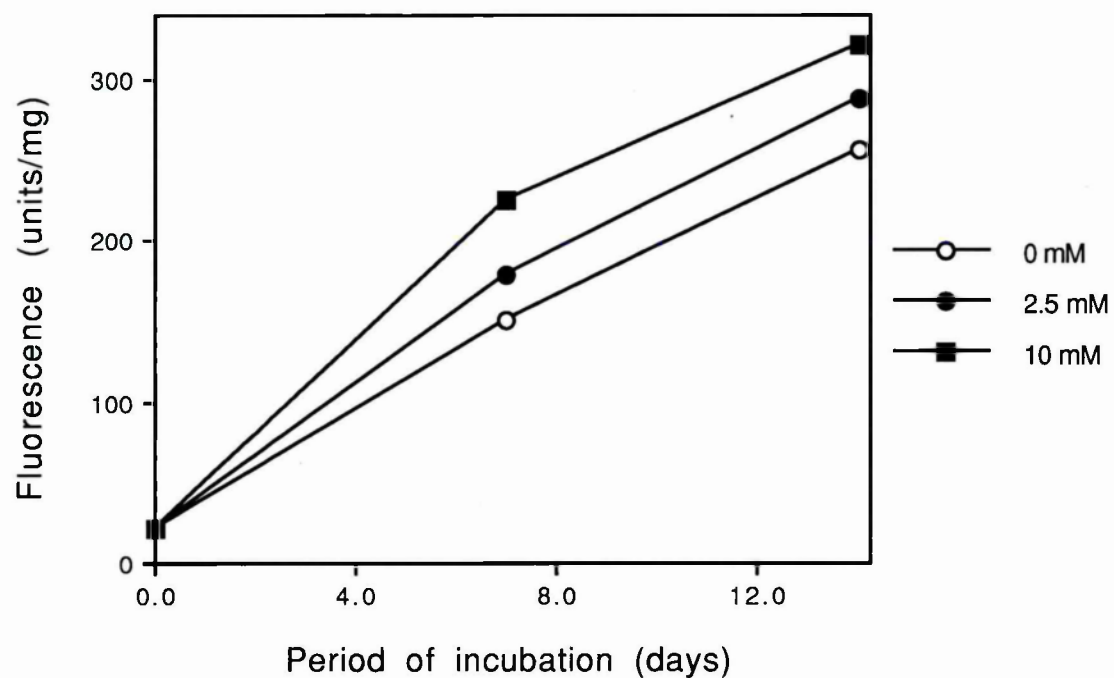


Fig 6.15: Effect of calcium on fluorescence of BSA (10mg/ml) incubated in 0.5M fructose

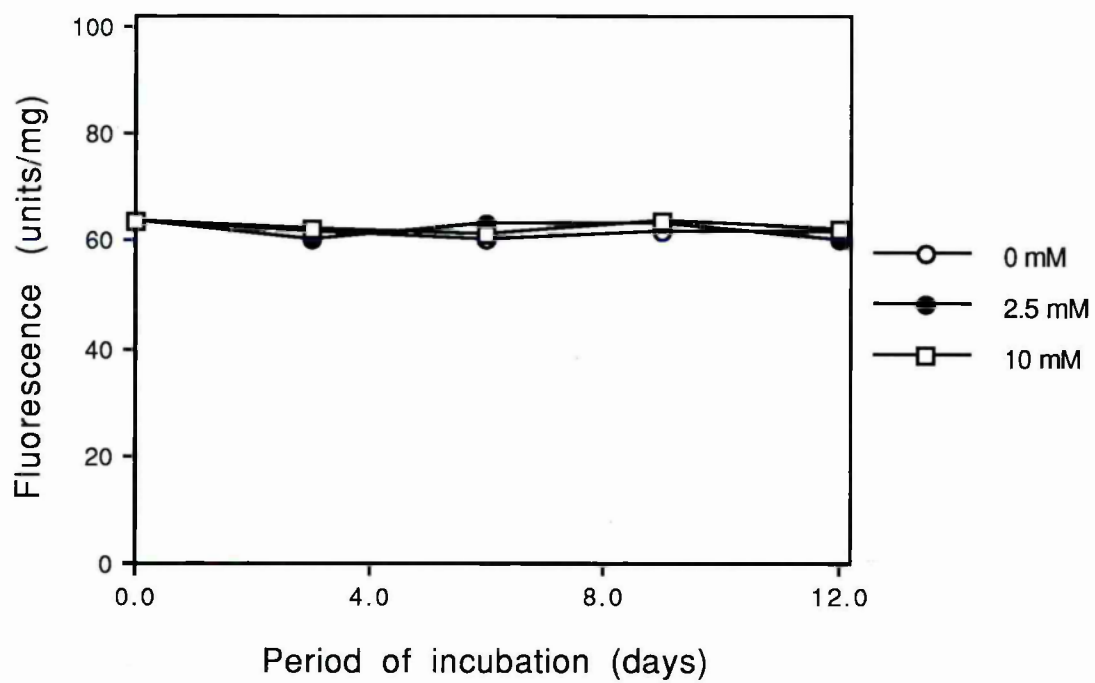


Fig 6.16: Effect of calcium on fluorescence of glucated BSA reincubated in the absence of sugar

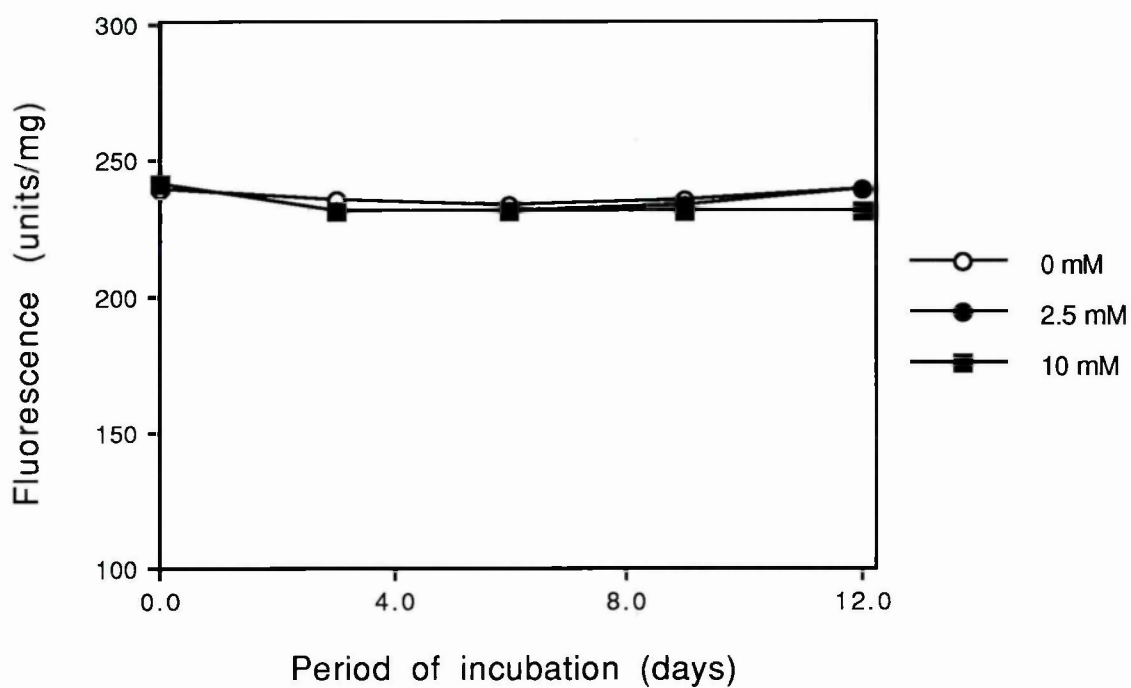


Fig 6.17: Effect of calcium on fluorescence of fructated BSA reincubated in the absence of sugar

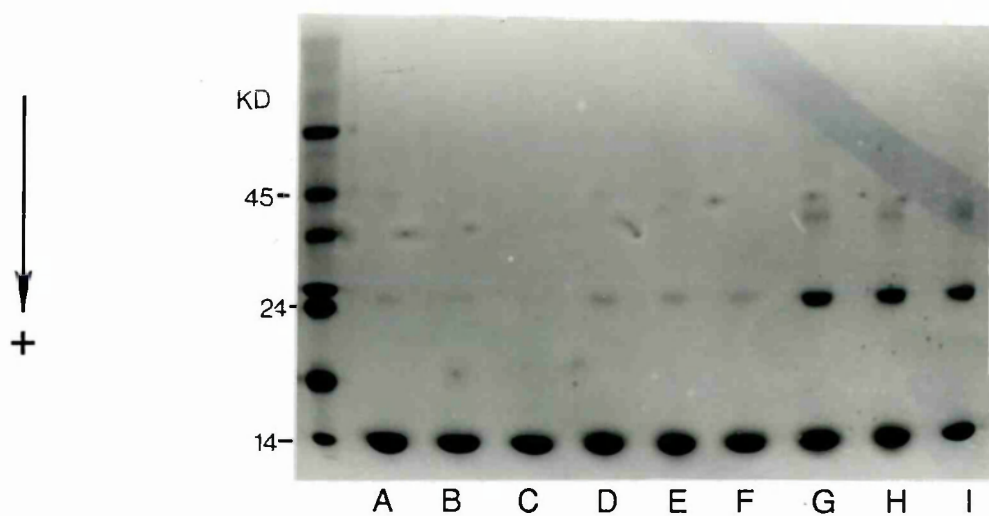


Fig 6.18: SDS-PAGE of lysozyme (lanes Band C), glucated lysozyme (lanes E and F) and fructated lysozyme (lanes H and I) incubated in 0 and 5mM calcium respectively for 9 days in the absence of sugar. Also shown is lysozyme (lane A), glucated lysozyme (lane D) and fructated lysozyme (lane G) before reincubation.

Protein sample	Time (days)	Calcium (mM)	% Oligomer
Lysozyme	0	0	0
Lysozyme	9	0	0
Lysozyme	9	5	0
Gluc lysozyme	0	0	4.3
Gluc lysozyme	9	0	3.4
Gluc lysozyme	9	5	4.1
Fruc lysozyme	0	0	24.3
Fruc lysozyme	9	0	24.0
Fruc lysozyme	9	5	26.4

Table 6.3: Effect of calcium on crosslinking of lysozyme, glucated lysozyme and fructated lysozyme reincubated in the absence of sugar

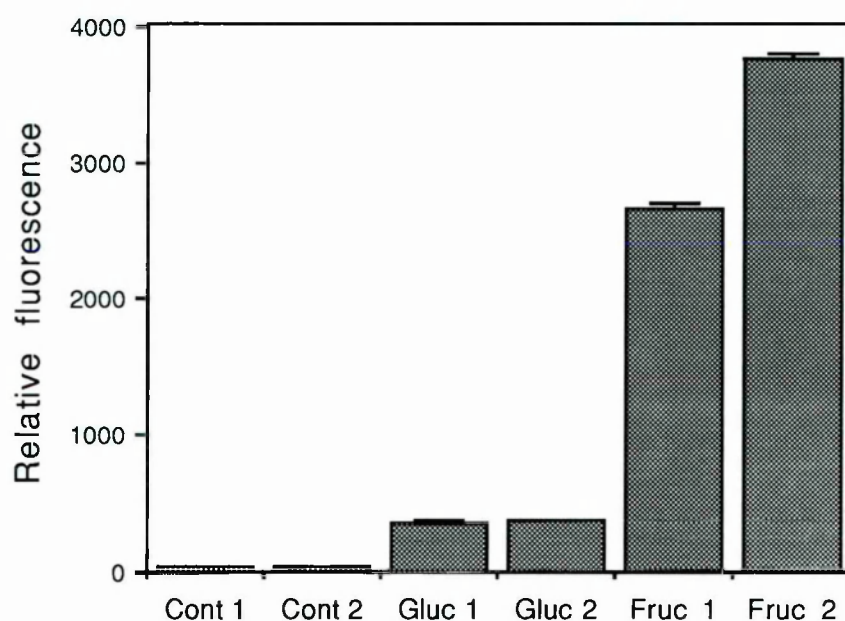


Fig 6.19: Fluorescence of BSA incubated alone (Cont 1), in 0.5M glucose (Gluc 1) or fructose (Fruc 1) before dialysis and after 4 days of dialysis (Cont 2, Gluc 2 and Fruc 2). Results are expressed as mean \pm SD for duplicate experiments.

6.5: Discussion

From my results, it is clear that phosphate (fig 6.1), pH (fig 6.7) and calcium (fig 6.13) have no effect on fluorescence of BSA alone and any increase is mediated via glycation. Other workers have shown that phosphate increases glucation of RNase by catalysing the Amadori rearrangement and this has been said to account for the higher glycation values obtained during in vitro glycation of proteins in phosphate buffers (164). The increased glycation may be in part due to greater reactivity of the sugar since phosphate also increases the proportion of sugar in the acyclic form (165). Here an increase in fluorescence of glucated and fructated BSA with phosphate has been demonstrated (figs 6.2 and 6.3 respectively). This increase is probably due to increased AP formation of BSA in phosphate and to increased secondary glycation (discussed in next chapter). However, the possibility that phosphate may be catalysing formation of intermediates in the Maillard reaction cannot be excluded. A small phosphate-catalysed increase in fluorescence is detectable for glucated BSA reincubated in the absence of sugars (fig 6.4) but not for fructated BSA (fig 6.5). In the case of glucated BSA, phosphate was required for the increase in fluorescence since incubation in a non phosphate buffer produced no change in fluorescence at pH 7.4 (fig 6.16). This compares with earlier work by McPherson et al who reported a significant rise in fluorescence of both glucated and fructated RNase upon reincubation in the absence of sugars in a phosphate buffer (57). However, Schleicher et al (90) and Kato et al (172) could not detect any appreciable rise in fluorescence upon reincubation of glucated albumin. Eble et al reported increased crosslinking of glucated RNase upon removal of sugars which was attributed to decreased competition between carbonyl groups from AP and glucose for free amino groups on protein (55). Using glycated lysozyme, no such increase in

crosslinking was demonstrable after removal of free sugar in this study (table 6.1). It is noteworthy that previous studies showing increases in crosslinking of glucated proteins upon removal of free sugar used RNase which was reincubated in phosphate buffer (55, 57). It may well be possible that only certain proteins are susceptible to crosslinking upon removal of unbound sugar.

Fluorescence of BSA incubated in glucose (fig 6.8) or fructose (fig 6.9) also increased with pH but only a small rise was noted for glucated BSA incubated in the absence of sugars (fig 6.10) whereas no change was detectable for fructated BSA (fig 6.11). An increase in crosslinking of glucated lysozyme is detectable upon reincubation in the absence of sugar and the reasons for this are not clear (table 6.2). Increased glycation at higher pH has been reported by several workers, and is due to a higher proportion of unprotonated amino groups and greater reactivity of sugar since a higher proportion exists in the acyclic form with increased pH. Both of these effects will increase AP formation which in turn increases AGE formation. However, the results for glucated BSA reincubated in phosphate buffer of increasing pH in the absence of sugar do suggest the possibility that conversion of AP to fluorophores may occur faster at a higher pH.

Calcium increases fluorescence of BSA incubated in glucose (fig 6.14) or fructose (fig 6.15) even at physiological concentrations, although the rise is not as marked as that with phosphate or pH. However it is not known whether this rise is statistically significant. Calcium has no effect on fluorescence of glycated BSA (figs 6.16 and 6.17) or crosslinking of glycated lysozyme (table 6.3) reincubated in the absence of sugar. The mechanism by which calcium may induce a rise in fluorescence of BSA incubated in sugars is not clear but it is

possible that binding of calcium ions to albumin may produce conformational changes in the protein increasing its susceptibility to glycation and fluorescence formation.

These studies suggest that free sugars are required for fluorescence and crosslinking of proteins and conflict with the findings of Eble et al (55) and McPherson et al (57) who have suggested that once a protein is glycated it will participate in AGE formation on return to normoglycaemia. However this in vitro study suggests that this may not be the case and may account for the improvement of certain diabetic complications upon strict metabolic control of glucose.

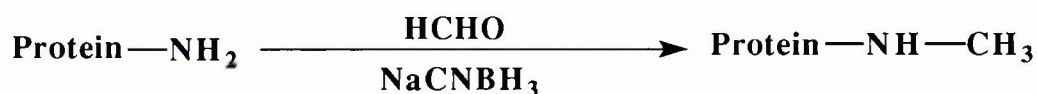
During dialysis of both glucated and fructated BSA, a rise in fluorescence occurs (fig 6.19) which is greater with fructated compared to glucated BSA. This suggests that the conversion of fructose-derived AP to AGE may reach saturation during dialysis hence the lack of fluorescence generation upon reincubation. Furthermore crosslinking has been reported to precede fluorophore formation and may have gone to completion during dialysis (over a period of 4 days). This may explain why glucated BSA still exhibits an increase in fluorescence but not crosslinking upon reincubation. A major limitation of the dialysis procedure is the time period required for adequate removal of unbound sugar. Over this period the Maillard reaction continues to proceed and important changes may have been masked thus a more rapid method would have been more appropriate.

Secondary Glycation of Proteins

7.1: Introduction

During in vitro glycation of proteins, fructose induces fluorescence considerably faster than glucose and this is believed to be due to two major reasons; (i) greater reactivity of fructose compared to glucose since a higher proportion exists in the acyclic form and (ii) faster conversion of fructose-derived AP (in particular those that are aldehydic in nature) to fluorophores probably due to their greater reactivity. Some workers have reported that removal of free sugar after glycation results in a faster increase in fluorescence (55, 57), however, our results in the previous chapter could not confirm these findings. It therefore seems that free sugar is actually required for fluorescence generation, particularly in the case of fructose.

The work in this chapter was carried out at the New York Medical College under the supervision of Dr Gerardo Suarez and evidence is presented for a reaction between free sugars and Amadori groups (named secondary glycation) which is responsible for generation of fluorescent products. In order to study the reaction between free sugars and AP, it was necessary to block any free amino groups in the glycated protein to prevent reinitiation of glycation. This process named reductive methylation can be outlined as;



Free amino groups from protein were blocked by methyl groups after treatment with HCHO and sodium cyanoborohydride (142).

This secondary glycation is particularly pronounced for fructose and may account for the higher fluorescence of fructated proteins. It also provides an explanation for the lack of fluorescence generated by glycated proteins upon reincubation in the absence of sugars.

7.2: Aims

- 1: To investigate whether free glucose can induce fluorescence of BSA by reaction with the AP .
- 2: To investigate whether free fructose can also do the same and how this compares with glucose.
- 3: To investigate how the above changes in fluorescence relate to the changes in free amino and Amadori groups.

7.3: Experimental Protocol

In Vitro Glycation: BSA (fatty acid free) at a concentration of 6mg/ml was incubated at 37°C in 0.5M glucose for 16 days in 0.05M sodium phosphate buffer, pH 7.0 under sterile conditions (section 2.1). The glucated BSA was dialysed exhaustively to remove free sugar (section 2.2) and stored frozen.

Reductive Methylation of Glucated BSA: The dialysed glucated BSA was reductively methylated to block free amino groups (section 2.5).

Reincubation of RM Glucated BSA: The reductively methylated glycated BSA was reincubated at 37°C in 0.1M glucose, fructose or in the absence of sugar in 0.05M sodium phosphate buffer, pH 7.0 for upto 25 days and stored frozen. Samples were dialysed (section 2.2) and stored frozen prior to any assays.

Protein Concentration: Protein was assayed according to the method of Lowry (section 2.4.1).

TNBS Assay: Free amino groups were assayed according to the TNBS assay (section 2.7).

Measurement of AP: The levels of AP were measured using the periodate assay according to the Gallop procedure (section 2.8.1).

Measurement of AGE: Samples were digested with proteinase K (section 2.6) before fluorescence measurements (section 2.9.1). These assays were also performed on samples prior to reincubation.

7.4: Results

Reductively methylated (RM) glucated BSA in the absence of sugars did not show an increase in fluorescence upon reincubation (fig 7.1). However, fluorescence increased in the presence of glucose and was higher with fructose (fig 7.1), clearly suggesting that free sugars are required for fluorescence of glycated proteins, and that fructose is more reactive in this respect. Prior to reincubation, the glucated BSA had 2.5 amino groups per mole and released 5.5 moles of HCHO per mole of protein. To show that this increase in fluorescence was not due to reinitiation of glycation, free amino groups were monitored over the reincubation period (fig 7.2), and no consumption of these groups was detectable. If Amadori products are required for formation of fluorophores, then consumption of these groups should occur with increase in fluorescence, and for this reason, they too were monitored. The moles of AP per mole of protein declined with period of incubation for all samples (fig 7.3). The decline in AP levels for RM-glucated BSA reincubated alone was only seen in this particular experiment and could not be reproduced. In a separate experiment, RM-glucated BSA reincubated alone shows no decline in AP levels whereas a decline is seen in the presence of glucose or fructose (fig 7.4).

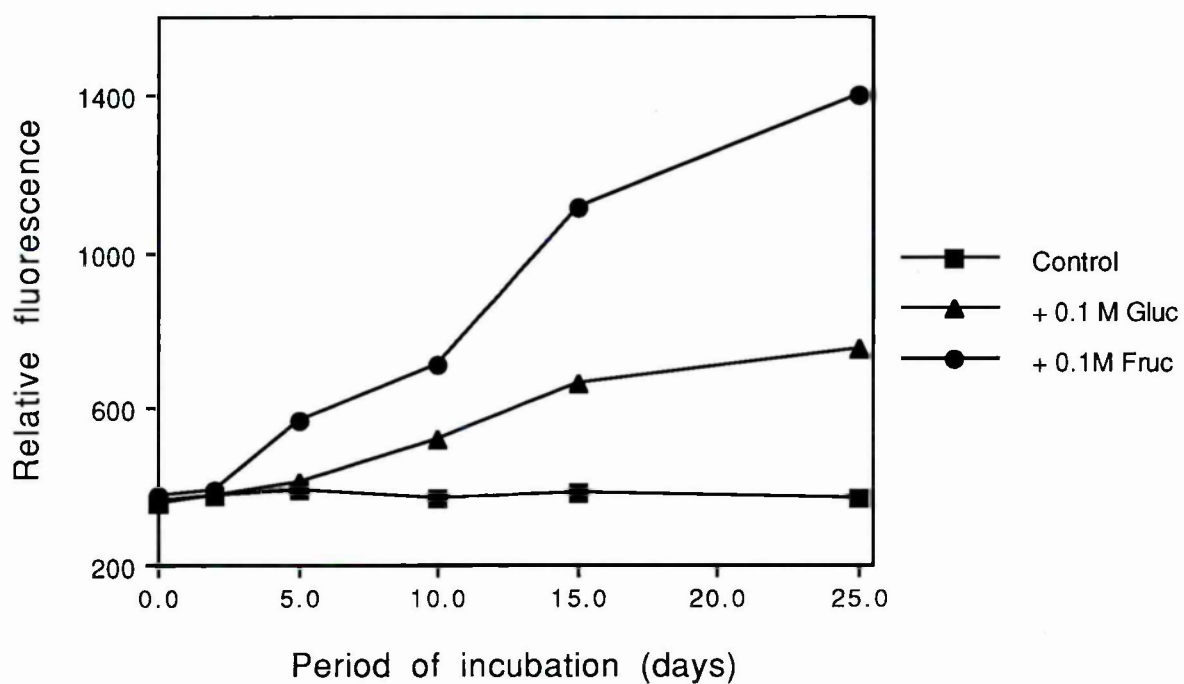


Fig 7.1: Fluorescence of RM-glucated BSA reincubated alone or in 0.1M sugar

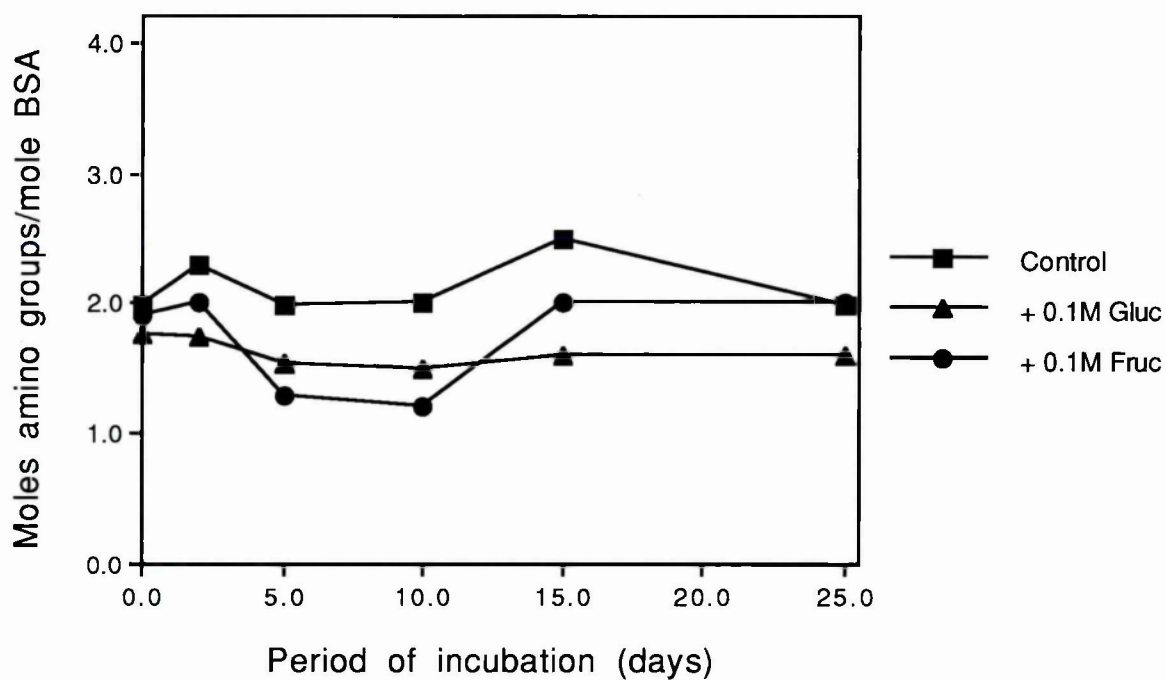


Fig 7.2: Free amino groups measured in RM-glucated BSA reincubated alone or in 0.1M sugar

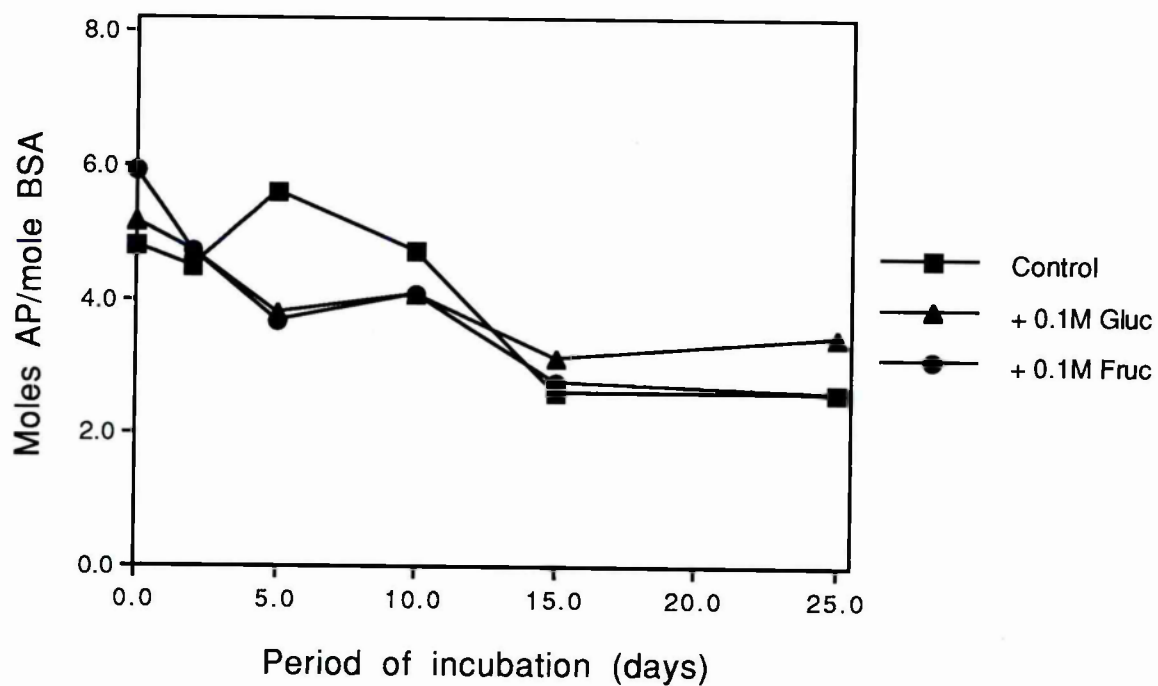


Fig 7.3: Apparent AP levels in RM-glucated BSA reincubated alone or in 0.1M sugar

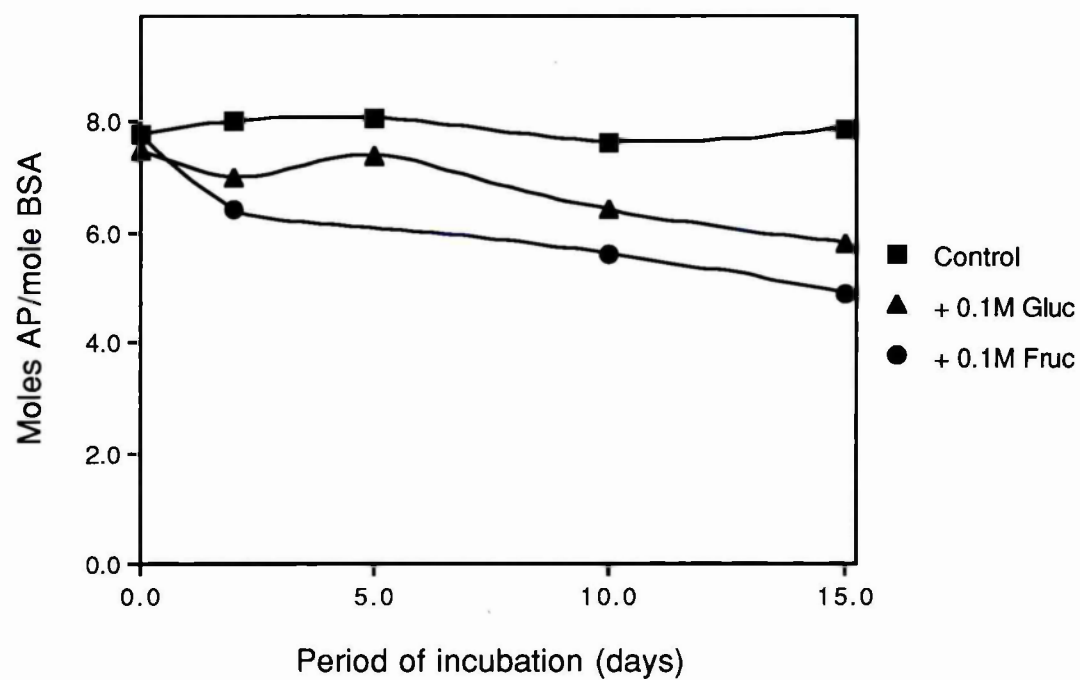


Fig 7.4: Apparent AP levels in RM-glucated BSA reincubated alone or in 0.1M sugar

7.5: Discussion

Glycated BSA was methylated to prevent further glycation occurring at the free amino groups. Sodium borohydride could have been used instead of cyanoborohydride since it is a stronger reducing agent. However, use of borohydride for reductive methylation may cause reduction of Amadori groups as well as peptide cleavage which will generate new free amino groups (142). Due to the high number of free amino groups within BSA, the process of reductive methylation had to be repeated (5-6 times) to achieve acceptable blocking of free amino groups. Complete blocking was not possible since even with cyanoborohydride, a small amount of fragmentation does occur. The RM-glucated BSA was then reincubated with sugars to study interaction between free sugar and the AP. Fluorescence measurements of RM-glucated BSA were made on proteinase K digests. This digestion was necessary because conformational changes induced by methylation may cause fluorescent groups within BSA to come into close proximity with quenching groups in other domains of protein causing a decline in fluorescence measured. Indeed, this was the case and was remedied by digestion of the protein. The RM-glucated BSA reincubated alone showed no increase in fluorescence, whereas an increase is seen in the presence of glucose or fructose (fig 7.1). This demonstrates that sugars are required for fluorescence formation which is considerably greater with fructose. This increase in fluorescence is not due to reinitiation of glycation since levels of free amino groups remain unchanged (fig 7.2). Furthermore, reinitiation of glycation should result in generation of AP which is not the case since a decline in AP is observed (fig 7.3). This consumption of AP may be accounted by their reaction with free sugars to generate fluorophores assuming that the sugar-AP adducts do not release HCHO after periodate oxidation. This reaction (named secondary glycation) is

faster with fructose leading to a higher generation of fluorescence and consumption of AP presumably because a greater proportion exists in the acyclic form compared to glucose. The consumption of AP in RM glucated BSA reincubated in the absence of sugar was unexpected, and the reason for this is not known. However it is worth pointing out that these experiments were repeated three times and in other experiments the AP levels in RM glucated BSA reincubated alone remained constant (fig 7.4). These results provide indirect evidence for the reaction of free sugars with AP producing fluorescent products. Dr G.Suarez has proposed a hypothetical pathway by which this reaction may occur (fig 7.5);

- (i) the free carbonyl group of fructose adds to the secondary amino group on AP via a nucleophilic addition reaction.
- (ii) the C-1 on added fructose could then combine with the C=O group of AP by aldol condensation so that the ring closes.
- (iii) double dehydration from this ring structure could then generate a pyrrole.

This pyrrolic structure may go on to form a fluorophore or may be fluorescent itself. Furthermore, this product has a carbonyl group and could participate in crosslink formation although this has not been investigated in this study. Pyrrolic structures have been demonstrated as intermediates in the Maillard reaction (17) and have been detected in vivo bound to collagen using Ehrlichs reagent (174). This secondary glycation may increase the fluorescence of previously glycated protein in vivo and may account for the lack of fluorescence generated by glycated proteins reincubated in the absence of sugars. Furthermore, it also accounts in part, for the high fluorescence during fructation compared to glucation.

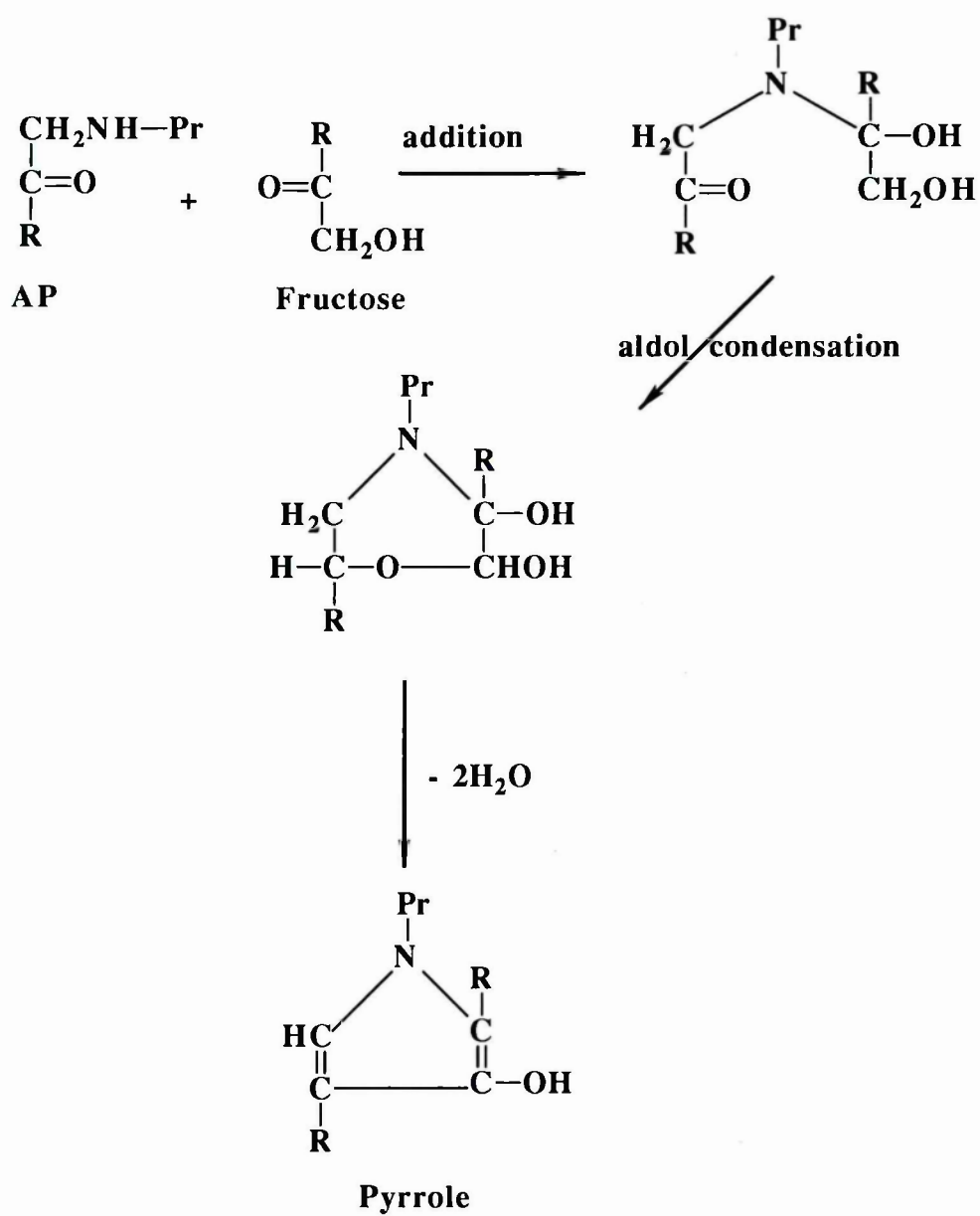


Fig 7.5: Secondary glycation of a protein by fructose

Inhibitors of Post-Amadori Reactions

8.1: Introduction

At present there is considerable interest in compounds which can inhibit AP formation or post-Amadori reactions because of their possible therapeutic potential in preventing the secondary complications of diabetes. Furthermore such compounds are of interest in food chemistry where they could be used to reduce or prevent undesirable changes in foods containing protein and sugars, particularly during storage. A number of compounds have been shown to inhibit glycation or AGE formation either *in vitro* or *in vivo*. The mechanisms by which these compounds act can be divided into three types;

- (i) blocking of free amino groups from proteins preventing them from being glycated or participating in AGE formation eg acetylsalicylic acid (127).
- (ii) blocking of free carbonyl groups from sugars, AP and reactive intermediates such as 3-DG eg aminoguanidine (122) and penicillamine (57).
- (iii) antioxidants and free radical scavengers may be used to inhibit AGE derived by autoxidative glycation eg vitamin E (134) and sorbitol (33).

Aminoguanidine has received the most attention and has been shown to reduce glycation (121) as well as fluorescent and crosslinked AGE formation *in vitro* (119). Comparative studies between aminoguanidine, pyridoxal phosphate and ascorbate have shown that aminoguanidine is the most effective at reducing *in vitro* glucation of serum albumin (175). In this work, equimolar concentrations of aminoguanidine and three other inhibitors, two of

which act by different mechanisms have been used to determine which is the most effective at reducing fluorescence and crosslinking of lysozyme. Since the mechanism of fructose-derived fluorescence and crosslinking of proteins may be different from that of glucose, the inhibitory effect of these compounds on fructose-derived changes to protein structure were also investigated. The three proposed inhibitors apart from aminoguanidine used in this study include acetylsalicylic acid, L-lysine and N-acetyl-L-cysteine.

Acetylation of proteins by acetylsalicylic acid has been reported to occur by two orders of magnitude faster than glycation (176) which is probably why this compound is an effective antiglycation agent. Unlike the other compounds acetylsalicylic acid is used clinically as an analgesic and patients with rheumatoid arthritis may have plasma levels of upto 200uM (177). I used this concentration of acetylsalicylic acid to determine whether it would be effective at reducing fluorescence of albumin exposed to levels of glucose found in diabetics.

L-lysine is a naturally occurring amino acid and its mechanism of inhibition should be similar to aminoguanidine. Previous studies by Sensi et al have demonstrated inhibition of glycation by D-lysine (178) and to my knowledge this is the first study to investigate the effect of L-lysine on sugar-derived AGE in proteins. If naturally occurring substances like lysine can reduce AGE formation, then it may be more appropriate to use these in vivo since they are likely to be less toxic than aminoguanidine. A problem with using L-lysine in vivo is that this amino acid is incorporated into proteins. This is not the case with D-lysine, but in vivo the D isomer can be readily converted to the L isomer (187).

Free radicals derived by sugar autoxidation can cause crosslinking and fluorescence of proteins and these changes are indistinguishable from those

that arise via the Amadori route. N-acetyl-L-cysteine is a free radical scavenger and should inhibit fluorescence and crosslinking of proteins by free radicals generated during sugar autoxidation. This compound was used to see whether free radicals produced during sugar autoxidation are of significance in producing the crosslinking and fluorescence changes.

The compound ortho-phenylenediamine can react with dicarbonyl compounds and has been used to isolate deoxyglucosones (179). In this study the effect of ortho-phenylenediamine on glucose and fructose-derived fluorescence and glycation of BSA was investigated. The effect of ortho-phenylenediamine on glucose and fructose-induced crosslinking of lysozyme was also investigated.

Whether any of the above compounds are of use in preventing diabetic complications remains to be seen, however, these compounds are of use as tools for studying mechanisms of glycation and AGE formation.

Aspects of this work, in particular the effect of phenylenediamine on BSA glycation and fluorescence was carried out at the New York Medical College, U.S.A. under the supervision of Dr Gerardo Suarez.

8.2: Aims

- 1: To compare the effects of equimolar concentrations of proposed inhibitors which act by different mechanisms to determine which type is the most effective at reducing glucose and fructose-derived fluorescence and crosslinking of lysozyme.
- 2: To investigate whether doses of acetylsalicylic acid achieved in vivo can protect against fluorescent-AGE formed on proteins exposed to levels of glucose found in diabetes.

- 3: To investigate the effect of phenylenediamine on glucose and fructose-derived glycation and fluorescence of BSA.
- 4: To investigate the effect of phenylenediamine on glucose and fructose-derived crosslinking of lysozyme.

8.3: Experimental Protocol

In Vitro Glycation: Lysozyme (10mg/ml) and BSA fatty acid free (6mg/ml) were incubated in 0.1M sugar (+/- 0.25-2.5mM of the stated inhibitor) for upto 7 days in 0.1 or 0.2M sodium phosphate/3mM sodium azide buffer, pH 7.4 at 37°C (section 2.1). HSA (40mg/ml) was incubated in 20mM glucose +/- 200uM acetylsalicylic acid in 0.05M sodium phosphate buffer/6mM sodium azide under the above conditions for upto 45 days. Samples were dialyzed to remove free sugar and inhibitors (section 2.2).

Protein Concentration: The protein was assayed using the Pierce BCA protein assay reagent (section 2.4.2).

Measurement of AP: The AP levels were measured using the periodate assay according to the Gallop procedure (section 2.8.1).

Measurement of AGE: The AGE were detected by their fluorescence (section 2.9.1) or crosslinking (section 2.9.2).

Statistical analysis was performed using the Students t test.

8.4: Results

Fig 8.1 shows the effect of acetylsalicylic acid on sugar-derived fluorescence of lysozyme. The fluorescence of lysozyme increases by about 50% when incubated with glucose and almost 8 fold in the fructose incubation. Fluorescence of lysozyme incubated in acetylsalicylic acid is much greater than in incubations of lysozyme with sugars alone. This acetylation of lysozyme

will therefore mask any inhibitory effect on sugar-derived fluorescence. Preliminary results show that lower concentrations of acetylsalicylic acid (200uM) inhibit glucose-derived fluorescence of HSA by 15% after 45 days of incubation. HSA incubated in acetylsalicylic acid alone appears to show an increase in fluorescence (fig 8.2). It is not known whether these changes are significant and this experiment needs to be repeated several times for statistically viable data.

Aminoguanidine reduces both glucose ($p<0.001$) and fructose-derived ($p<0.001$) fluorescence of lysozyme (fig 8.3). Glucose-derived fluorescence of lysozyme is reduced to that generated by non-glycated lysozyme after incubation with aminoguanidine whereas fructose-derived fluorescence is inhibited by over 70%.

L-lysine causes a significant reduction of both glucose ($p<0.01$) and fructose-derived fluorescence of lysozyme ($p<0.001$) (fig 8.4).

The free radical scavenger N-acetyl-L-cysteine decreases glucose-derived fluorescence of lysozyme ($p<0.05$) but not fructose-derived fluorescence (fig 8.5).

When the effect of these compounds on crosslinking was examined, aminoguanidine proved to be the most effective in the order aminoguanidine > acetylsalicylic acid > L-lysine > N-acetyl-L-cysteine (figs 8.6 and 8.7 and table 8.1).

Phenylenediamine was found to have no effect on AP formation of BSA as shown in table 8.2. However, there was significant reduction of both glucose and fructose-derived fluorescence of BSA ($p<0.001$). Glucose-derived fluorescence of BSA was reduced by 29% and 45% in incubations with 0.25mM and 0.50mM phenylenediamine respectively (fig 8.8). For fructose-induced fluorescence, the corresponding reductions were 35% and 48% (fig

8.9). An increase in fluorescence of BSA incubated alone with phenylenediamine also occurs despite extensive dialysis to remove any unbound phenylenediamine (figs 8.8 and 8.9).

Phenylenediamine reduced crosslinking of both glucated and fructated lysozyme in a dose dependent manner (fig 8.10). The ability of phenylenediamine to inhibit crosslinking of fructated lysozyme appears to be greater than its ability to inhibit crosslinking of glucated lysozyme (table 8.3).

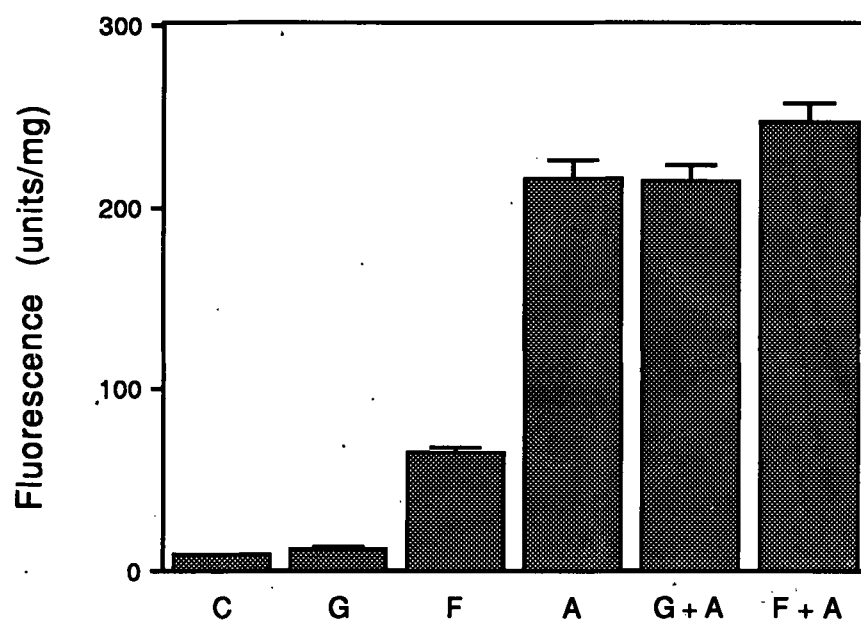


Fig 8.1: Fluorescence of lysozyme (10mg/ml) incubated for 7 days either alone (C), in 0.1M glucose (G) or fructose (F). The effect of 2.5mM acetylsalicylic acid on fluorescence of lysozyme incubated alone (A), in 0.1M glucose (G+A) or fructose (F+A) is also shown. Results are expressed as mean \pm S D (n=5).

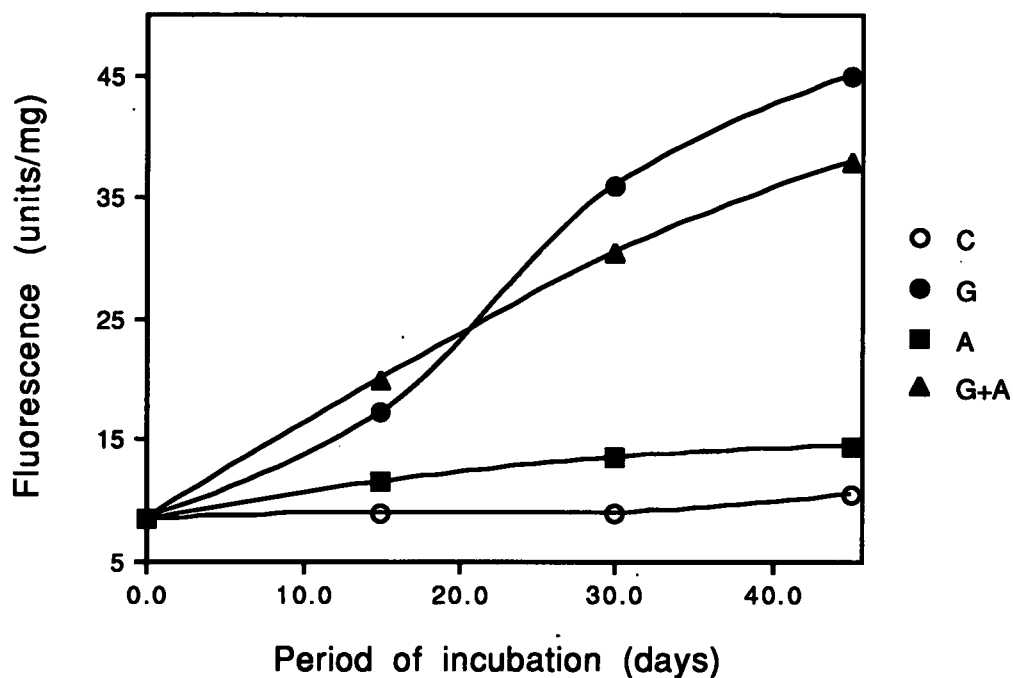


Fig 8.2: Fluorescence of HSA (40mg/ml) incubated either in the absence (C) or presence of 20mM glucose (G). The effect of 200uM acetylsalicylic acid on fluorescence of HSA incubated in the absence (A) or presence of 20mM glucose (G+A) is also shown.

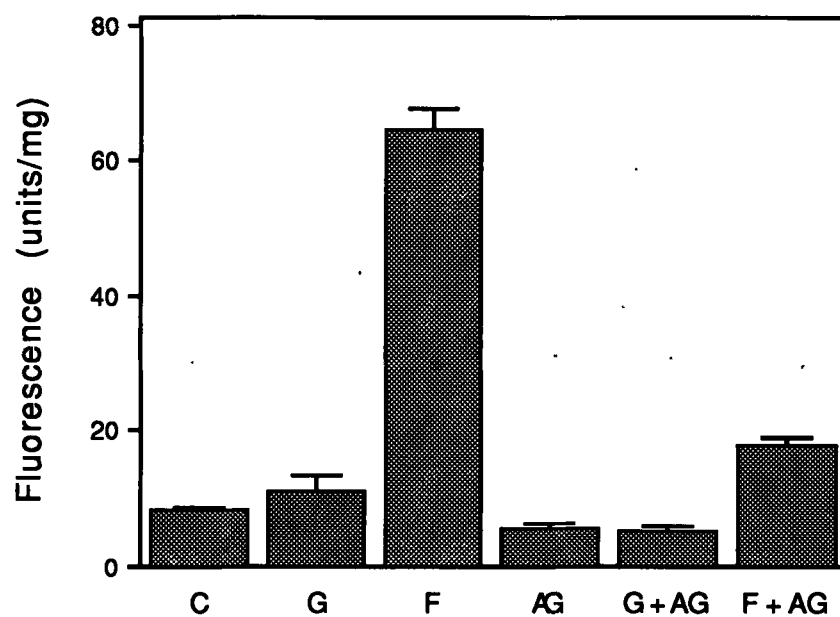


Fig 8.3: Fluorescence of lysozyme (10mg/ml) incubated for 7 days either alone (C), in 0.1M glucose (G) or fructose (F). The effect of 2.5mM aminoguanidine on fluorescence of lysozyme incubated alone (AG), in 0.1M glucose (G+AG) or fructose (F+AG) is also shown. Results are expressed as mean \pm SD (n=5).

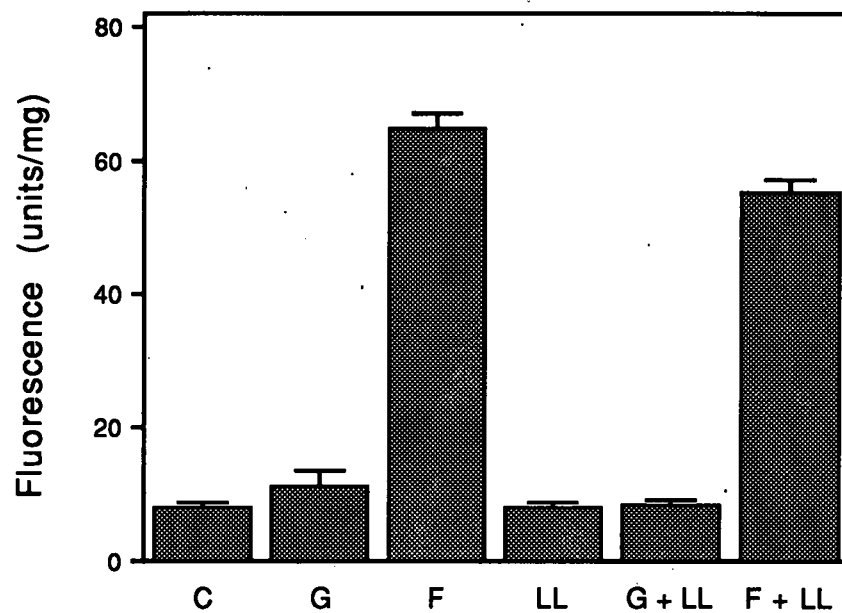


Fig 8.4: Fluorescence of lysozyme (10mg/ml) incubated for 7 days either alone (C), in 0.1M glucose (G) or fructose (F). The effect of 2.5mM L-lysine on fluorescence of lysozyme incubated alone (LL), in 0.1M glucose (G+LL) or fructose (F+LL) is also shown. Results are expressed as mean \pm SD (n=5).

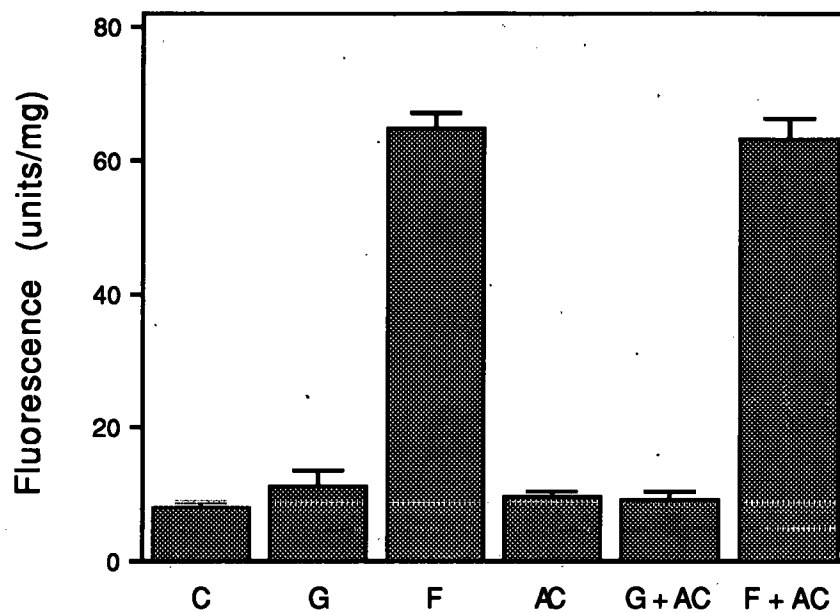


Fig 8.5: Fluorescence of lysozyme (10mg/ml) incubated for 7 days either alone (C), in 0.1M glucose (G) or fructose (F). The effect of 2.5mM N-acetyl-L-cysteine on fluorescence of lysozyme incubated alone (AC), in 0.1M glucose (G+AC) or fructose (F+AC) is also shown. Results are expressed as mean \pm SD (n=5).

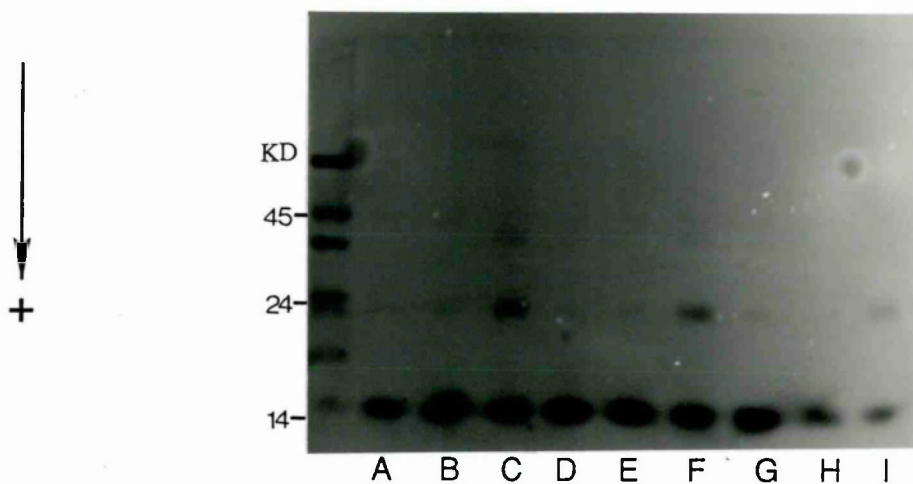


Fig 8.6: SDS-PAGE of lysozyme (10mg/ml) incubated in the absence (lane A) or presence of 0.1M glucose (lane B) or fructose (lane C) for 7 days. The effect of 2.5mM acetylsalicylic acid and aminoguanidine on lysozyme incubated in the absence (lanes D and G) or presence of 0.1M glucose (lanes E and H) or fructose (lanes F and I) respectively are also shown.

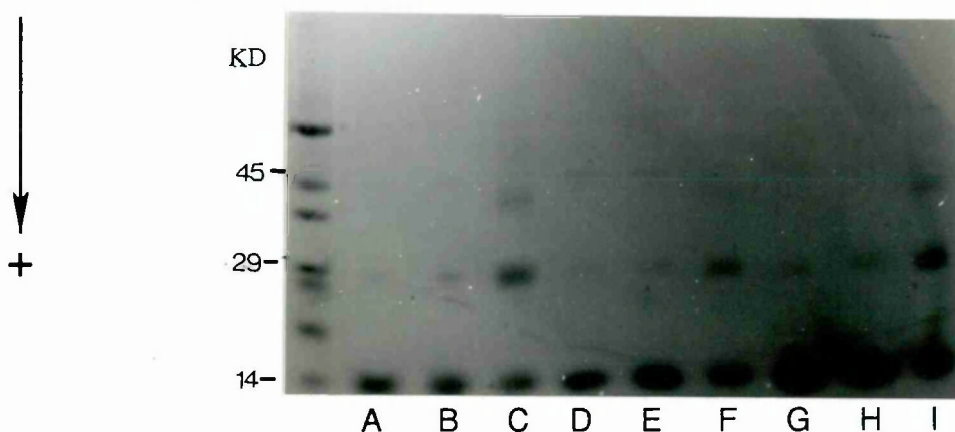


Fig 8.7: SDS-PAGE of lysozyme (10mg/ml) incubated in the absence (lane A) or presence of 0.1M glucose (lane B) or fructose (lane C) for 7 days. The effect of 2.5mM L-lysine and N-acetyl-L-cysteine on lysozyme incubated in the absence (lanes D and G) or presence of 0.1M glucose (lanes E and H) or fructose (lanes F and I) respectively are also shown.

Protein	Sugar	Inhibitor	% Oligomer
Lysozyme	None	None	0
Lysozyme	Glucose	None	1.3
Lysozyme	Fructose	None	14.5
Lysozyme	None	ASA	0
Lysozyme	Glucose	ASA	0
Lysozyme	Fructose	ASA	7.4
Lysozyme	None	Aminoguanidine	0
Lysozyme	Glucose	Aminoguanidine	0
Lysozyme	Fructose	Aminoguanidine	2.3
Lysozyme	None	L-lysine	0
Lysozyme	Glucose	L-lysine	0.7
Lysozyme	Fructose	L-lysine	10.5
Lysozyme	None	NALC	0
Lysozyme	Glucose	NALC	1.0
Lysozyme	Fructose	NALC	18

Table 8.1: Effect of 2.5mM acetylsalicylic acid (ASA), aminoguanidine, L-lysine or N-acetyl-L-cysteine (NALC) on crosslinking of lysozyme (10mg/ml) incubated for 7 days in the absence or presence of 0.1M sugar in 0.1M phosphate.

Protein	Sugar	PD	Moles AP/mol
BSA	None	None	0.33
BSA	None	0.25mM	0.30
BSA	None	0.50mM	0.34
BSA	Glucose	None	1.95
BSA	Glucose	0.25mM	1.95
BSA	Glucose	0.50mM	1.98
BSA	Fructose	None	0.52
BSA	Fructose	0.25mM	0.46
BSA	Fructose	0.50mM	0.45

Table 8.2: Effect of phenylenediamine (PD) on apparent AP levels in BSA (6mg/ml) incubated for 7 days in the absence or presence of 0.1M sugar.

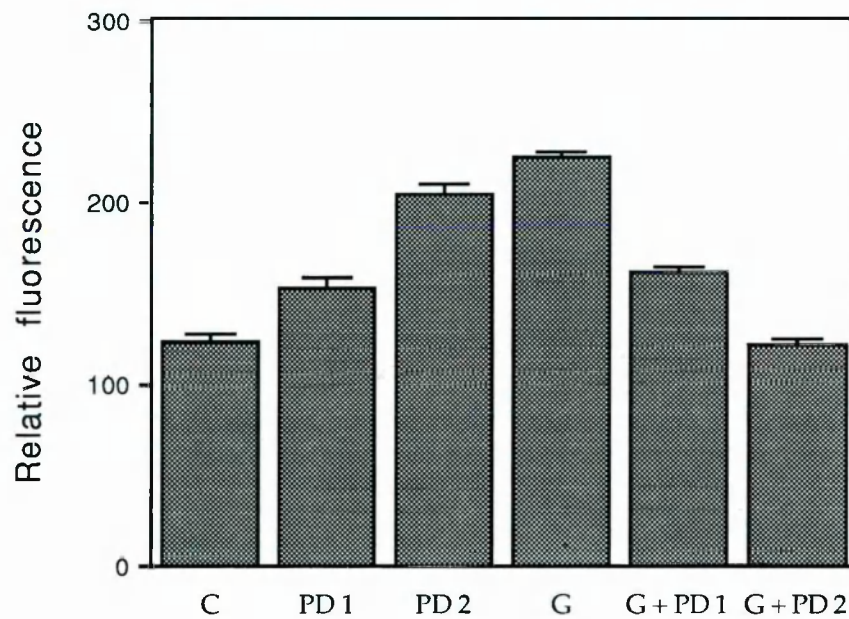


Fig 8.8: Fluorescence of BSA (6mg/ml) incubated for 7 days either alone (C) or in 0.1M glucose (G). The effect of phenylenediamine on fluorescence of BSA incubated either alone (PD 1, PD 2) or in 0.1M glucose (G+PD 1, G+PD 2) is also shown where PD 1 is 0.25mM and PD 2 is 0.50mM phenylenediamine. Results are expressed as mean \pm SD of duplicate experiments.

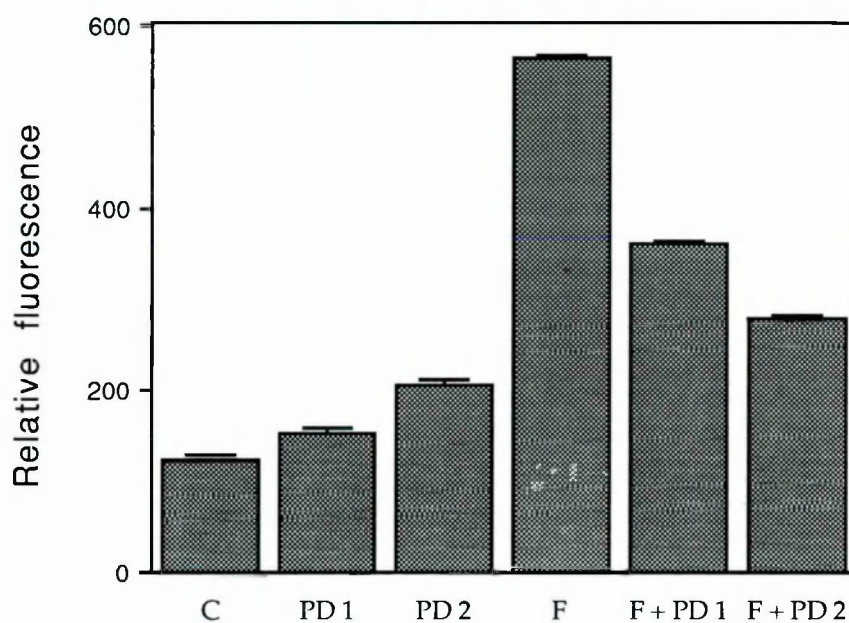


Fig 8.9: Fluorescence of BSA (6mg/ml) incubated for 7 days either alone (C) or in 0.1M fructose (F). The effect of phenylenediamine on fluorescence of BSA incubated alone (PD 1, PD 2) or in 0.1M fructose (F+PD 1, F+PD 2) is also shown where PD 1 is 0.25mM and PD 2 is 0.50mM phenylenediamine. Results are expressed as mean \pm SD of duplicate experiments.

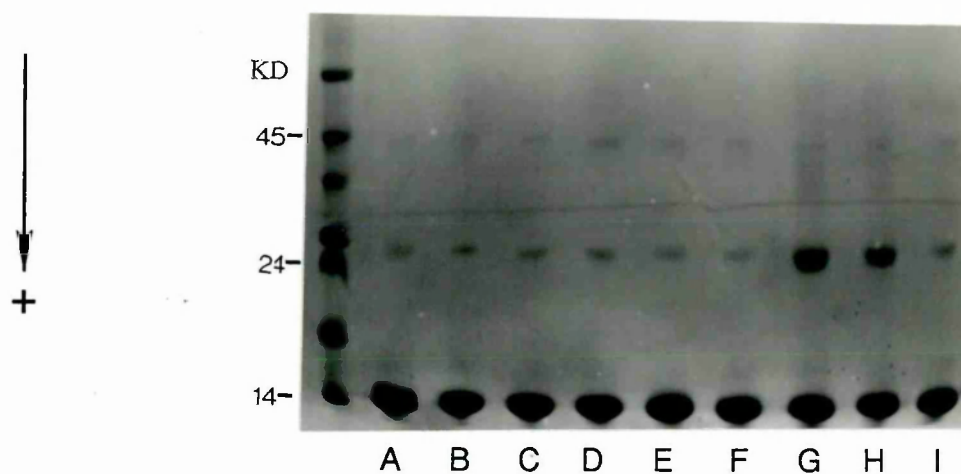


Fig 8.10: SDS-PAGE of lysozyme (10mg/ml) incubated for 7 days in (i) the absence of sugar with 0, 0.25 and 2.5mM phenylenediamine (lanes A, B and C respectively), (ii) 0.1M glucose with 0, 0.25 and 2.5mM phenylenediamine (lanes D, E and F respectively) and (iii) 0.1M fructose with 0, 0.25 and 2.5mM phenylenediamine (lanes G, H and I respectively).

Protein	Sugar	P D	% Oligomer
Lysozyme	None	None	0
Lysozyme	None	0.25mM	0.7
Lysozyme	None	2.50mM	0.9
Lysozyme	Glucose	None	3.4
Lysozyme	Glucose	0.25mM	3.0
Lysozyme	Glucose	2.50mM	2.3
Lysozyme	Fructose	None	24.1
Lysozyme	Fructose	0.25mM	14.1
Lysozyme	Fructose	2.50mM	6.8

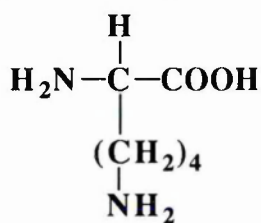
Table 8.3: Effect of phenylenediamine (PD) on crosslinking of lysozyme (10mg/ml) incubated for 7 days in the absence or presence of 0.1M sugar in 0.2M phosphate.

8.5: Discussion

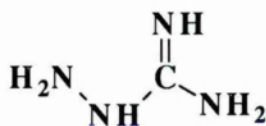
Acetylation of proteins by acetylsalicylic acid has been reported to have a protective effect against glycation *in vitro* (126, 127). Whether such a protective effect occurs *in vivo* is not yet clear although acetylsalicylic acid feeding has been shown to reduce crosslinking of lens crystallins in diabetic rats (132). Acetylation may induce a conformational change in the protein structure and this might account for the high fluorescence observed with incubations containing acetylsalicylic acid (fig 8.1). Furthermore, acetylsalicylic acid may itself be fluorescent, and indeed acetylsalicylic acid on its own does fluoresce. It is therefore not possible to compare the ability of acetylsalicylic acid to reduce sugar-derived fluorescence of lysozyme with the other compounds. Use of lower concentrations of acetylsalicylic acid may overcome these problems. In an attempt to simulate *in vivo* conditions, a 200uM concentration of acetylsalicylic acid was used and a small inhibitory effect on glucose-derived fluorescence of HSA is demonstrable (fig 8.2). A rise in fluorescence of albumin incubated in acetylsalicylic acid also occurs but this is probably masked by the higher glucose-derived fluorescence. From these *in vitro* studies it seems that levels of acetylsalicylic acid approached *in vivo* do not cause a substantial reduction in glucose-derived protein fluorescence.

Under the conditions used, aminoguanidine proved to be the most effective inhibitor of glucose and fructose-derived fluorescence of lysozyme (fig 8.3). There are a variety of compounds present *in vivo* which possess free amino groups and such compounds may act in a similar way to aminoguanidine and could therefore be used instead of aminoguanidine since they are presumably less toxic. For this reason, L-lysine was also included in the study, and although an inhibitory effect could be demonstrated (fig 8.4), it was not as

effective on a molar basis as aminoguanidine. The relative nucleophilicities and pK values of amino groups in inhibitors are important and may determine their reactivity. The pK of lysine is 10.4 whereas the pK of aminoguanidine does not appear to be known. Since aminoguanidine has a similar structure to arginine (ie both have a guanidino group), then its pK may be close to that of arginine ie 12.5. The chemical structures of lysine and aminoguanidine are given below;



lysine



aminoguanidine

Aminoguanidine is a hydrazine compound and two of its amino groups are chemically more reactive than those of lysine (120) and this probably accounts for its greater inhibitory effect.

Free radicals generated by sugar autoxidation have been reported to induce fluorescence of proteins (33, 36, 39). To determine whether this free radical-derived fluorescence contributes substantially towards the total fluorescence generated, a free radical scavenger ie N-acetyl-L-cysteine was also included but had the least effect on fluorescence of lysozyme incubated alone or in sugars (fig 8.5). These studies therefore suggest that fluorescence changes induced by sugars are due to buildup of AGE derived via the Amadori route since free radical scavengers appear to have little effect on glucose or fructose-derived fluorescence.

Aminoguanidine is also the most effective compound at reducing crosslinking of lysozyme (fig 8.6 and table 8.1). In contrast to the inconclusive fluorescence

studies, here it is clear that acetylsalicylic acid also reduces crosslinking of lysozyme but not to the same extent as aminoguanidine. This difference may be because aminoguanidine not only inhibits post-Amadori reactions but can also react with free carbonyl groups from sugars preventing glycation ie can act at more sites than acetylsalicylic acid. It is worth pointing out that amines like aminoguanidine could theoretically reduce secondary glycation by reacting with free sugars. Decreased secondary glycation of proteins would be accompanied by decreased fluorescence and possibly crosslinking, however this has yet to be proved. The free radical scavenger N-acetyl-L-cysteine has the least effect on sugar-derived crosslinking of lysozyme (fig 8.7 and table 8.1) which suggests that free radicals do not have a significant role in sugar-induced crosslinking under the conditions used.

A possible explanation for the increase in fluorescence of BSA incubated with phenylenediamine is the non covalent binding of the diamine to protein. Such binding probably also occurs with glycated BSA but is masked by their greater fluorescence. Theoretically phenylenediamine could also react with free carbonyl groups from reducing sugars or AP therefore reducing glycation and AGE production respectively. However, the compound has no effect on glycation of BSA (table 8.2) which suggests that it reacts with post-Amadori intermediates ie 3-DG. It is unlikely that the diamine reacts with free carbonyl groups from AP since this would affect HCHO release upon periodate oxidation, which is not the case. Phenylenediamine also reduces crosslinking of lysozyme, particularly when incubated with fructose (table 8.3). Fructation induces 3-DG formation almost twice as fast as glucation (155), and since the diamine is reacting with these, this may explain its greater inhibitory effect on fructose-derived crosslinking.

Autoxidative Glycation and Protein Fragmentation

9.1: Introduction

Exposure of proteins to sugar in vitro for prolonged periods of time results in protein crosslinking and fluorescence. Such changes to the protein structure in vivo has been implicated in the pathogenesis of diabetic complications. A recent hypothesis proposed by Hunt et al suggests that autoxidation of glucose catalysed by transition metals generate ketoaldehydes and free radicals which contribute towards the changes in protein structure mentioned above (33). Furthermore, the hydroxyl radical generated during autoxidative glycation can induce protein fragmentation. Hunt et al have demonstrated such fragmentation in vitro by incubating BSA with 25mM glucose in the presence and absence of 100uM Cu^{2+} (fig 1 of ref (33)). BSA incubated in the absence of glucose showed no fragmentation suggesting that the process is sugar-induced. Fragmentation of BSA has a specific pattern in that polypeptide fragments of 45, 36 and 29KD are produced. Inhibition of glucose-derived fragmentation by the metal chelator DETAPAC lead to the suggestion that the process is transition metal catalysed.

In the previous chapters, no fragmentation was detectable for BSA, lysozyme or RNase on SDS gels. This lack of fragmentation could be due to the fact that gels were stained with Coomassie Blue R which may not be sensitive enough to detect any fragmentation. Furthermore, all the incubations contained sodium azide, a potential free radical scavenger. To see whether fructose and glucose can induce protein fragmentation, I used HSA as a model protein and

incubated it under identical conditions used by Hunt et al (33). Fragmentation was examined using silver stained gels to increase sensitivity by a 100 fold. To determine whether azide inhibits fragmentation, HSA was incubated in glucose in the absence and presence of azide.

Copper ions stimulate protein fragmentation by increasing production of the hydroxyl radical responsible for fragmentation (35). Iron will also catalyse production of hydroxyl radicals but unlike copper does not bind to albumin. Fragmentation of albumin in the presence of copper but not iron will suggest that binding of metal to protein may be important for glucose-derived fragmentation. Different concentrations of copper and iron were included in incubations to see whether there was a dose dependent effect on both glucose-derived fragmentation and fluorescence.

9.2: Aims

- 1: To investigate glucose and fructose-derived fragmentation of HSA incubated under the identical conditions of Hunt et al.
- 2: To investigate the effect of different concentrations of copper and iron on glucose-derived fragmentation of HSA.
- 3: To investigate the effect of sodium azide on glucose-derived fragmentation of HSA incubated in the presence or absence of copper or iron.
- 4: To investigate the effect of different concentrations of copper and iron on glucose-derived fluorescence of HSA.

9.3: Experimental Protocol

In Vitro Glycation: HSA fatty acid free (1mg/ml) was incubated in 25mM sugar +/- 50-200uM cupric sulphate or iron chloride in 0.1M potassium phosphate buffer, pH 7.4 at 37°C for 8 or 16 days. Incubations contained 3mM sodium azide or were performed under sterile conditions (section 2.1). Samples were not dialysed.

Detection of Fragmentation: Protein fragmentation was examined on 10% SDS gels after silver staining (section 2.9.2).

Measurement of AGE: Fluorescence was measured as before (section 2.9.1).

Statistical analysis was performed using the Students t test.

9.4: Results

HSA was glycated under the exact conditions used by Hunt et al but no bands equivalent to the polypeptide fragments in fig 1 of ref (33) were detectable after 8 or 16 days of incubation (fig 9.1, lanes A to D)). Similarly incubations with fructose do not give rise to any fragmentation products either (fig 9.1 lanes E and F).

The protein readily fragments in the presence of 50-200uM copper (lanes B to D, fig 9.2). However the fragmentation pattern produced is not like that obtained by Hunt et al in that discrete polypeptide fragments ranging from 45-29KD are not detectable. Iron appears to have little effect on fragmentation of HSA suggesting that binding of the metal to protein may well be required for fragmentation (lanes F to H, fig 9.2). Since sodium azide may act as a free radical scavenger, the above were repeated in the absence of azide and no difference was detectable (fig 9.3) suggesting that azide does not have an inhibitory effect on fragmentation.

The effect of different concentrations of copper on fluorescence of HSA is shown in fig 9.4. A significant rise in fluorescence of HSA occurs after addition of 50uM copper ($p<0.001$). Further increases in the levels of copper upto 200uM result in a significant reduction of fluorescence ($p<0.01$). In contrast, no significant increase in glucose-derived fluorescence of HSA occurs after addition of 50uM iron but a significant increase does occur with 100 and 200uM concentrations of iron ($p<0.001$) as shown in fig 9.5.

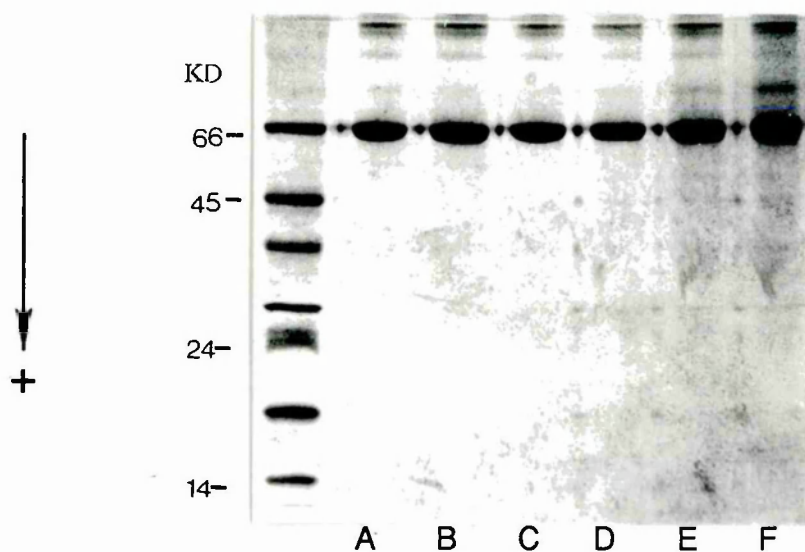


Fig 9.1: SDS-PAGE of HSA (1mg/ml) incubated in the absence of sugar (lanes A and B) or in 25mM glucose (lanes C and D) or fructose (lanes E and F) for 8 and 16 days respectively.

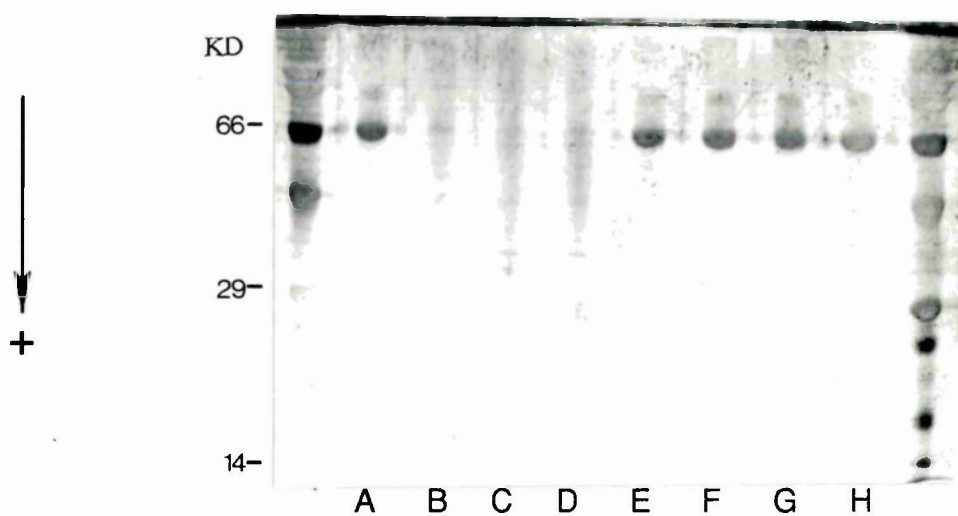


Fig 9.2: SDS-PAGE of HSA (1mg/ml) incubated for 8 days in 25mM glucose in the presence of azide. The effects of 50uM (lanes B and F), 100uM (lanes C and G) and 200uM (lanes D and H) copper and iron respectively are shown.

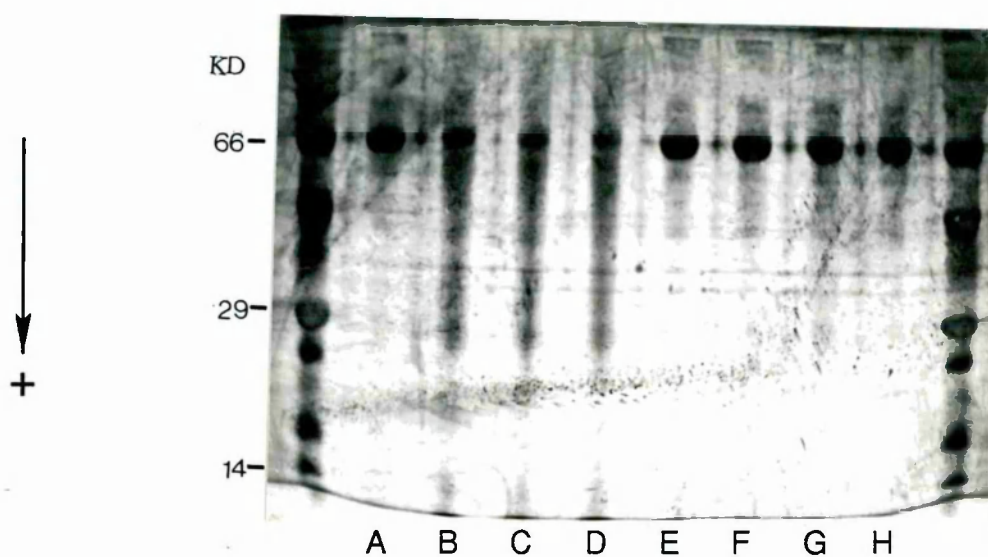


Fig 9.3: SDS-PAGE of HSA (1mg/ml) incubated for 8 days in 25mM glucose in the absence of azide. The effects of 50uM (lanes B and F), 100uM (lanes C and G) and 200uM (lanes D and H) copper and iron respectively are shown.

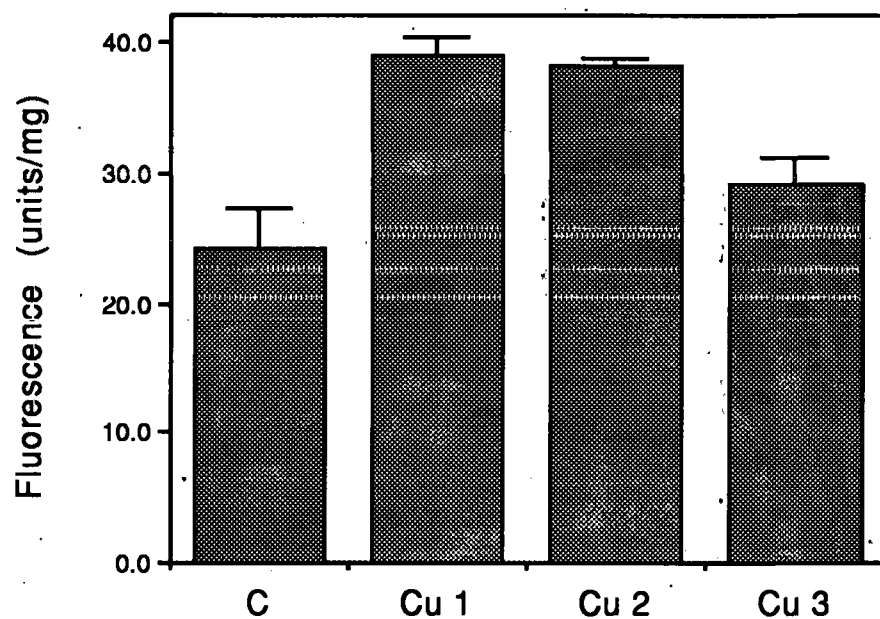


Fig 9.4: Fluorescence of HSA (1mg/ml) incubated in 25mM glucose (C) for 8 days in the absence of azide. The effect of different concentrations of copper is also shown where Cu 1 is 50uM, Cu 2 is 100uM and Cu 3 is 200uM. Results are expressed as mean \pm SD (n=5).

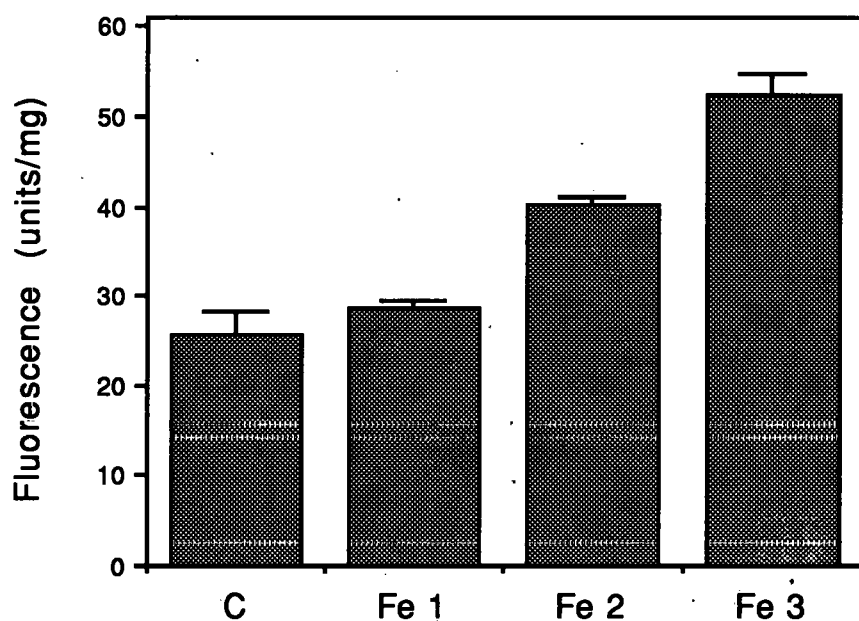


Fig 9.5: Fluorescence of HSA (1mg/ml) incubated in 25mM glucose (C) for 8 days in the absence of azide. The effect of different concentrations of iron is also shown where Fe 1 is 50uM, Fe 2 is 100uM and Fe 3 is 200uM. Results are expressed as mean \pm SD (n=5).

9.5: Discussion

In an attempt to reproduce the sugar-derived fragmentation pattern of Hunt et al, proteins were incubated under the exact conditions used by this group except that azide was included to prevent bacterial growth. However, I was unable to demonstrate any fragmentation of HSA incubated in glucose or fructose in the absence of transition metals (fig 9.1). This finding therefore disagrees with that of Hunt et al who demonstrated fragmentation even in the absence of transition metals (fig 1, ref (33)). In support of my results other workers too have not detected any fragmentation in the absence of transition metal ie Sakurai et al (54), Dominiczak, and Kawakishi (personal communication).

To demonstrate that the above result was not due to the possible free radical scavenging ability of azide, incubations were also performed without azide ie under sterile conditions. Glucose-derived fragmentation in the absence of transition metals still could not be detected and the azide appeared to have no effect (figs 9.2, and 9.3). However fragmentation was readily detectable in incubations containing copper and appeared to be in a dose dependent manner because careful examination of the gel shows a reduction in intensity of the 66KD band for HSA with increasing copper concentrations (fig 9.2). Hunt et al produced a specific pattern for BSA fragmentation which was attributed to the fact that copper binds to histidine residues in albumin. Autoxidation and hydroxyl radical production at these sites may produce the polypeptide fragments with molecular weights of 45, 36 and 29KD. However I was unable to detect such a specific pattern for fragmentation. Furthermore incubations containing iron showed little fragmentation of HSA. This suggests that specific binding of the transition metal may be required for oxidative cleavage of the

protein. Indeed more recent work by Hunt et al shows less than 2% fragmentation of lens crystallin (180) compared to over 10% for BSA (33).

Hunt et al have reported that physiological buffers already contain trace amounts of transition metals which are in excess of that required to stimulate glucose autoxidation and ketoaldehyde production (36). Addition of further transition metal did not therefore increase ketoaldehyde production but did increase fragmentation (181) since the latter is dependent on hydroxyl radical production. A possible explanation for the fragmentation seen by Hunt et al for BSA incubated in glucose alone (fig 1 ref (33)) could be contamination of their buffers with trace amounts of transition metal sufficient to stimulate glucose autoxidation. In support of this is the fluorescence results in fig 9.4 where addition of 50uM copper increases fluorescence presumably due to ketoaldehyde production since the buffer used in this study may not have sufficient transition metal to catalyse glucose autoxidation. In contrast, addition of copper in the study by Hunt et al was found to decrease fluorescence of BSA and was attributed to degradation of ketoaldehydes by excess transition metals thus lowering their steady state levels (33). Further increase in copper (200uM) also decreased fluorescence in this study (fig 9.4). Addition of iron however increases fluorescence in a dose dependent manner (fig 9.5).

From the above studies it appears that glucose autoxidation catalysed by copper does induce protein fragmentation. Plasma copper levels do rise during diabetes probably by an increase in ceruloplasmin, but even then whether these levels are sufficient to catalyse significant sugar-induced fragmentation is not yet known. Furthermore, sugar-induced fragmentation may not be a significant process in vivo since binding of copper is restricted to a limited number of proteins. To my knowledge there is no evidence at present to

suggest that fragmentation of proteins susceptible to diabetic complications does occur in vivo.

Overall Observations

10.1: Overall Discussion

The purpose of this chapter is to summarise and provide an overview of the work done in this thesis and to see how the individual topics interrelate with each other ie the experimental work will be considered as a whole rather than a series of individual topics.

In recent years glycation and the Maillard reaction have received considerable interest because of their possible role in the secondary complications of diabetes and ageing. These secondary complications are a major cause of morbidity and mortality in diabetics and the elderly and this justifies the continuous research into glycation. However, research in this field has been hampered due to several reasons. Not least is the lack of specific, sensitive and reproducible assays for quantifying glycation and Maillard products. Of the simple assays for glycation, the periodate assay of Gallop et al has the advantage in that it is stoichiometric, suitable for absolute values and can be used on intact proteins (43). In this work the method of Gallop et al has been modified and adapted for use with a microplate reader. This microassay has the advantage in that it is faster, more sensitive and has an improved yield of DDL. A major disadvantage of the periodate method is that collagen crosslinks interfere and so the assay is unsuitable for measuring glycated collagen. Although collagen was not investigated in this study, a microassay for glycated collagen was developed based on the TBA method of Parker et al (144). This TBA microassay is more sensitive, faster, less cumbersome than the Parker method and is suitable as a rapid assay for measuring glycated collagen. An

excellent correlation coefficient of 0.94 was obtained between the periodate microassay and the more established TBA method.

Almost all the glycation assays are designed to detect the glucose-derived AP because in vivo most proteins are likely to be glycated by this sugar. However glycation by other sugars, notably fructose is also possible, particularly during diabetes. I have confirmed that fructose-induced changes to protein structure occur considerably faster than those induced by glucose on a molar basis yet apparent AP levels measured by the periodate microassay for fructated BSA were consistently lower than those for glucated BSA. It is possible to account for this by considering the underlying chemistry of the assay and the nature of the fructose-derived AP. The highly reactive aldehydic AP derived during fructation is unlikely to release HCHO during periodate oxidation and so the true extent of fructation may be underestimated by this assay. Furthermore, it appears that this is true for other methods such as the TBA, fructosamine and phenylboronate assays (chapter 5, table 5.1). It can be argued that faster conversion of fructose-derived AP to AGE may be responsible for the apparent low levels of AP observed in fructated proteins when compared to glucated proteins. However ribose induces AGE formation considerably faster than fructose for albumin yet also has higher AP levels when measured by the periodate microassay (chapter 4, fig 4.1). It therefore seems that the true extent of protein damage by fructose may be underestimated by assays which measure the AP only. In contrast methods which measure the rate of SB and AGE formation clearly suggest the greater hazards of any in vivo fructation. A common feature of all AP is the possession of a carbonyl group and an assay which measures these may provide a better estimation of glycation. For this reason the DNPH assay was developed and the levels of protein bound carbonyl groups for fructated albumin were found to be considerably higher

than those for the corresponding glucated sample (chapter 5, fig 5.5). Therefore the DNPH assay gives a more accurate measure of fructose-derived changes to protein structure than any of the other assays used. This work has therefore revealed the inadequacies in present glycation assays and improved assays for glycation by both glucose and fructose have been developed.

At present there are hardly any simple, specific assays for AGE since very little information is available regarding their structure. The limited information available suggests that there are different types of AGE and methods used to quantify the few that have been characterised are available only in certain laboratories. In this study AGE were measured by their fluorescence and crosslinking properties. These procedures are not very specific because fluorescence and crosslinking of proteins can be caused by other processes notably free radicals and lipid peroxidation products (reviewed in (182)).

In vitro studies using radiolabelled sugars have demonstrated that nearly all the monosaccharides form the SB faster than glucose (11) and in vivo the low levels of these sugars could be compensated for by their greater reactivity. In this study the effect of different sugars on fluorescence formation was studied and it was found that ribose, fuctose and galactose induced fluorescence faster than glucose on a molar basis irrespective of the protein used (chapter 4, figs 4.2, 4.4 and 4.5). These comparative fluorescence studies are consistent with the comparative rates of SB formation ie of the four sugars used ribose, which would be expected to have the fastest rate of SB formation, was also found to have the fastest rate of fluorescence formation.

This thesis has also shed light on the mechanisms involved in AGE formation. Previous work has suggested that sugar-derived fluorescent products are formed by reaction between (i) two AP's, (ii) 3-DG and free amino groups or (iii)

3-DG and AP's. These products may or may not be crosslinks but are all believed to be fluorescent. In this study evidence has been presented for a possible reaction between the secondary amino group on AP and free sugars to produce fluorescent products. Whether these products give rise to crosslinks has not yet been determined. Furthermore, fluorescent products formed by secondary glycation depend on the reactivity of the glycating sugar, hence fructose induces secondary glycation faster than glucose. That secondary glycation is a significant process for fluorescence generation can be demonstrated by reincubating glycated proteins in the absence of sugar. Hardly any further increase in fluorescence is detectable in fructated BSA (chapter 6, fig 6.5) and only a small increase occurs in glucated BSA (chapter 6, fig 6.4). This small rise in fluorescence of glucated BSA incubated in the absence of sugar could be due to production of 3-DG from AP which then react with free amino groups to form protein bound pyrroles. This mechanism has been proposed for the formation of pyrraline (20). It is noteworthy that RM-glucated BSA reincubated in the absence of sugar does not show a further increase in fluorescence presumably because the amino groups are blocked and therefore not available for pyrrole formation (chapter 7, fig 7.1). In the case of fructated BSA, conversion of AP to AGE occurs so rapidly that the reaction may have already gone to completion and this may account for the lack of increase in fluorescence generated by fructated BSA upon reincubation in the absence of sugar. Another study by McPherson et al demonstrated a rise in fluorescence of both glucated and fructated RNase upon reincubation after removal of sugars (57). However these workers used a rapid procedure for removal of unbound sugar unlike the extensive dialysis over a period of 3-4 days used in this study.

Phosphate may catalyse conversion of AP to AGE since the rise in fluorescence of glucated BSA after removal of sugars only occurs in phosphate and not HEPES buffer. Phosphate is believed to increase percentage sugar in the acyclic form (165) and a similar effect on AP's which largely exist in the cyclic form may account for the rise in fluorescence of glucated BSA reincubated in phosphate buffer after removal of free sugars. In this study the fluorescence of glucated BSA reincubated in the absence of sugar also increased with pH. Like phosphate an increase in pH can also increase the percentage sugar in acyclic form (163) and a similar effect on the AP may account for the rise in fluorescence of glucated BSA. However this is mere speculation and as far as I know there is no information on the effect of pH or phosphate on the percentage of AP in acyclic form. Previous studies have shown that a rise in both phosphate (164) and pH (41) can increase the extent of glycation. In this study, phosphate and pH were found to increase fluorescence of both glucated and fructated BSA. This effect is mediated partly by increased glycation and probably increased secondary glycation as well.

Previous studies have shown that less than 1% of crosslinks in glucated lysozyme are fluorescent and the pathways for fluorescent and non-fluorescent crosslinks may operate independently of each other (26). Using equimolar concentrations of lysozyme and RNase incubated in ribose, I was able to show that RNase generates fluorescence considerably faster than lysozyme (chapter 4, fig 4.6) whereas the latter crosslinked slightly faster (chapter 4, fig 4.9). Furthermore the comparative rates of crosslinking by different sugars for lysozyme and RNase were not consistent with their comparative rates for fluorescence or SB formation. In this case it was found that fructose-derived crosslinking of RNase occurred almost as fast as that derived by ribose

whereas for lysozyme, fructose-derived crosslinking was actually faster than that induced by ribose. In contrast to the fluorescence studies for reincubated glucated BSA, no increase in crosslinking of fructated or glucated lysozyme was detectable after reincubation in the absence of sugar. The two studies which have demonstrated increased crosslinking of glycated proteins after removal of free sugars used RNase (55, 57), and to my knowledge this effect has not been demonstrated for other proteins.

Based on the studies of Eble et al (55) and McPherson et al (57) it is believed that once a protein is glycated it will continue to form AGE albeit at an accelerated rate even after removal of sugar. This is thought to be because the AP is no longer competing with free sugar carbonyls in order to react with protein amino groups. This finding has been used to explain why certain diabetic complications progress even after strict metabolic control. For eg it has been reported that diabetic retinopathy continues to progress in patients whose glycaemia is normalised after a successful pancreas transplantation (183). However the results in this study support those of Schleicher et al (90) and Kato et al (172) who too could not detect any appreciable rise in fluorescence of albumin after exhaustive removal of sugars. Using glycated lysozyme, no further rise in crosslinking was detectable after removal of free sugars. In contrast to the clinical studies above, there are other studies which have shown that strict metabolic control can reverse progression of certain diabetic complications. For eg strict metabolic control has been shown to reduce thickening of basement membranes (184) in diabetics and an improvement in diabetic neuropathy (185). These findings can be used to reiterate the importance of maintaining normoglycaemia in an attempt to reduce the risk of development and progression of diabetic complications.

An alternative to strict metabolic control would be to use drugs which could reduce glycation and AGE formation in vivo. In this respect aminoguanidine has received considerable interest as a possible inhibitor of AGE formation in vivo and at present is undergoing toxicity studies (120). In this study aminoguanidine proved to be the most effective inhibitor of glucose and fructose-derived fluorescence and crosslinking of lysozyme when compared on a molar basis with acetylsalicylic acid, L-lysine and N-acetyl-L-cysteine. A possible reason for this could be because it can act at more sites in the pathway for AGE formation. L-lysine may also act at these sites but is not as effective as aminoguanidine because of the lower reactivity of its amino groups.

In this study, phenylenediamine was used to inhibit glucose (chapter 8, fig 8.8) and fructose-derived fluorescence of BSA (chapter 8, fig 8.9). Similarly phenylenediamine inhibited glucose and fructose-derived crosslinking of lysozyme in a dose dependent manner. This compound probably reacts with 3-DG since it has no effect on glycation (chapter 8, table 8.2).

For a substance to be of any use as a inhibitor of glycation and AGE formation in vivo, it should have a long half life because glycation is a spontaneous reaction and occurs all the time. Furthermore the substance should have virtually no toxicity since it will probably have to be administered for long periods of time. Obviously all these requirements are not easy to fulfill and in my opinion it seems unlikely that administration of antiglycation or anti-AGE compounds will be of use in vivo for the prevention of diabetic complications.

A recent hypothesis proposed by Hunt et al has questioned whether the fluorescence and crosslinking changes produced during incubation of proteins in sugars is due solely to post-Amadori reactions (33). This group has

suggested that autoxidation of sugars may occur to produce reactive dicarbonyl compounds and free radicals which can cause protein fluorescence and crosslinking. Changes to protein structure as a result of autoxidative glycation are similar to those derived by glycation and it is very difficult to distinguish between the two. However the hydroxyl radical produced during autoxidative glycation can cause fragmentation of proteins. In this study, no evidence for fragmentation was seen even after using the exact conditions of Hunt et al (33). It was however possible to induce fragmentation by using copper which suggests that transition metals are required for autoxidative glycation. Furthermore binding of transition metal to protein is important since little fragmentation was detectable for HSA incubated in iron. The free radical scavenger N-acetyl-L-cysteine appeared to have little effect on fluorescence (chapter 8, fig 8.5) and crosslinking (chapter 8, table 8.1) of lysozyme suggesting that under the conditions used, sugar-derived free radicals do not contribute significantly towards these changes.

Suggestions for Further Study

If time permitted I would have liked to extend aspects of the work covered in this thesis. There are 4 major areas which merit further investigation;

- 1: The DNPH microassay used in this work gives higher values for protein bound carbonyl groups in fructated compared to glucated BSA. However it is not yet clear whether the majority of these carbonyl groups are from AP or AGE. Periodate treatment of proteins will selectively remove AP without affecting AGE (99). Values for protein bound carbonyl groups in fructated proteins before and after periodate oxidation should yield useful information regarding the contribution of AP carbonyl groups.
- 2: Studies in this work on fluorescence and crosslinking of glycated proteins after removal of free sugars were complicated by the long periods of dialysis prior to reincubation. These experiments could be repeated using a rapid method for removal of unbound sugar such as Sephadex G-25 columns.
- 3: The possibility of fluorophores being generated as a result of secondary glycation has been demonstrated in this work. However it is not known whether these secondary glycation products are also crosslinks. This work can therefore be repeated and crosslinking of the secondary glycated protein can be examined on SDS gels. The possibility that aminoguanidine may reduce fluorescence and crosslinking by inhibiting secondary glycation can also be investigated by including this compound in the incubations.
- 4: The secondary glycation products are believed to be pyrroles in nature and to confirm whether this is true, the method of Scott et al based on the

Ehrlich's reagent (174) could be used to assay for protein bound pyrroles in secondary glycosylated proteins.

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